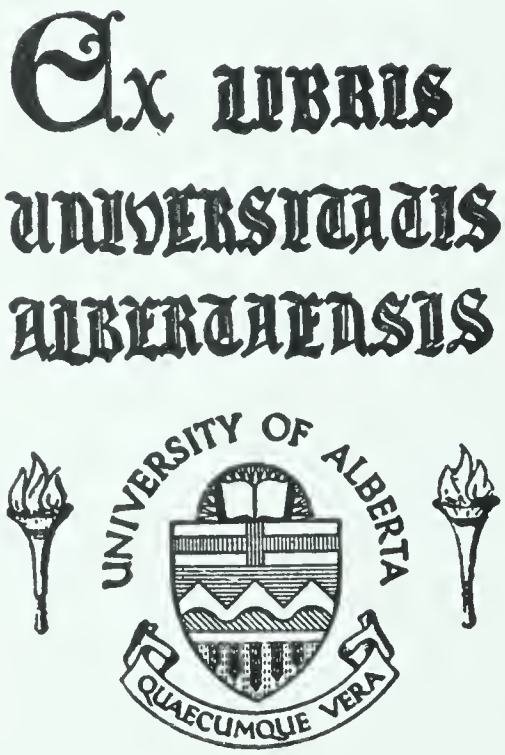

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CYTOTOXIC ISOANTIBODIES AGAINST CELLS OF THE RETICULO-ENDOTHELIAL SYSTEM IN INBRED MICE

by

Joan Amelia Stratton

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

APRIL, 1964

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cytotoxic Isoantibodies against Cells of the Reticulo-endothelial System in Inbred Mice," submitted by Joan Amelia Stratton, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Cytotoxic antisera were produced in inbred mice by the intraperitoneal injection of allogenic spleen cells suspended in Freund adjuvant. Fresh spleen cells were found to be more effective than lyophilized spleen cells in stimulating the production of isoantibodies. The cytotoxic isoantibodies appear to be directed against the strong H-2 histocompatibility antigens. The antibodies formed are specific for the H-2 antigens on the cells which elicited their production and they will cross react with other cells possessing some of these H-2 antigens.

Cytotoxic isoantisera will agglutinate allogenic mouse red blood cells *in vitro* when titrated by the Gorer and Mikulska human serum-dextran hemagglutination technique. The antibodies will also combine with H-2 antigens of the surface membrane of homologous spleen cells. In the presence of complement, the permeability of surface membranes is altered and the cells become swollen by the entrance of water and sodium ions from the medium. Potassium ions, protein, amino acids, ribonucleic acid and ribonucleotides are lost from the cells. The spleen cells also become permeable to a number of vital dyes, such as eosin Y, trypan blue and lissamine green.

Not all of the tissues of the adult reticulo-endothelial system are equally sensitive to the cytotoxic action of specific isoantibody. Notably thymus cells are almost completely resistant, while spleen and lymph node cells are very sensitive. Tissue cells from newborn mice are insensitive to the effects of cytotoxic isoantibodies. Adult levels of sensitivity are not reached until 7 to 9 weeks after birth. Certain cell types in the adult spleen show differential sensitivity to cytotoxic antiserum. The smallest cells are the most resistant and the medium-sized cells the most sensitive. The larger cells show no differential sensitivity or resistance.

Complement from a heterologous source is essential for the demonstration, *in vitro*, of the cytotoxic activity of

isoantisera. Very high concentrations of guinea pig serum are toxic to the mouse spleen cells. Low dilutions of mouse antiserum and normal serum are anti-complementary. A complement dilution of 1:8 provides an adequate excess of active complement and at this level the natural guinea pig anti-mouse antibody is diluted out. Complement fixation by the cytotoxic isoantibody system can be accurately measured by the addition of a rabbit anti-sheep hemolysin-sheep red blood cell indicator system. As the percent relative mortality of the spleen cells increases, so does the fixation of complement. The peak of complement fixation coincides well with the peak percent relative mortality. A second isoantibody(s) appears to be present in the cytotoxic antisera studied. This isoantibody(s) is anti-complementary at low dilutions and is complement-fixing. Although the isoantibody(s) fixes complement in the presence of normal spleen cells, it is not lethal to these cells.

Splenic nodules can be produced in lethally irradiated mice by the intravenous injection of isogenic spleen cells. An intravenous dose of approximately 10^4 cells is required for the production of one nodule. The number of splenic nodules or colonies formed bears a linear relationship to the number of injected cells. Cytotoxic isoantiserum inhibits splenic colony production, either by differentially killing those cells which are capable of colony formation, or by rendering cells ineffective in colony formation without altering their permeability to vital dyes in the *in vitro* titrations. Fifty-eight percent of the colonies appeared to be clonal in nature.

ACKNOWLEDGEMENTS

The author wishes to extend her appreciation to Dr. G. O. Bain, Department of Pathology, for supervision and guidance during the course of the investigations and in preparation of the thesis.

Appreciation is also extended to Dr. E. J. K. Penikett and the staff of the Department of Bacteriology, for some of the materials used in the investigations, and to Dr. T. K. Shnitka for the preparation of the electron-micrograph.

Grateful acknowledgement is extended to Dr. R. G. Moffat, Dr. S. R. Usiskin and the technical staff of the Cancer Clinic, for their cooperation and assistance in the irradiation of the mice.

The author is very grateful to Mrs. V. V. Scott for willing technical assistance, to Mrs. S. Ellis for typing the initial drafts of the thesis, to Miss M. Anderson for assistance with the graph layouts, to Mr. E. Beamont for processing the photomicrographs, and to Mr.J. Berg for his willing donations of blood which provided serum for the human serum-dextran hemagglutination titrations.

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I. INTRODUCTION

An antibody is a modified serum globulin, secreted into the circulation by cells of the plasmocytic series, as a consequence of the contact of these cells with an antigenic substance (95,121). Antibody globulins have the same physical and chemical properties as normal globulins (95). They are thought to differ from normal globulins by possessing areas (combining sites) which have a configuration complementary to that of the antigenic grouping with which they react (59). Antibodies are specific; that is, they react specifically with the substances used to produce them. However, they may cross-react with antigens showing a very close chemical relationship to the homologous antigen (59,90,95,121).

A cytotoxic isoantibody is an antibody produced in one strain of animal (recipient) by the injection of cells from another strain (donor), which, in the presence of active complement, is lethal to the cells of the donor. Interest in cytotoxic antibodies to mouse antigens began in 1936 when Gorer (38) demonstrated antibodies "I", "II", and "III" using a saline agglutination technique in a rabbit anti-mouse serum--mouse red blood cell system. In 1937, Lumsden demonstrated that malignant cells could be destroyed by the action of specific antibody and complement. However, it was Gorer (39,40) who first described

the appearance of specific isoantibodies in the sera of mice which had rejected tumor homografts. On the basis of serological data, he postulated the genes that govern the fate of tumor and normal tissue homografts. The isoantibodies detected by a saline agglutination technique were weak, capricious and transient. The difficulties encountered in the demonstration of isoantibodies were reduced considerably by the introduction of the human serum-dextran hemagglutination technique of Gorer and Mikulska (47). This highly sensitive method increased the regularity with which H-2 isoantibodies could be detected and was useful in the study of genetic configurations. Even the human serum-dextran method had limitations, for some of the histocompatibility (H-2) components are absent, or at least present in very small quantities on the surface of mouse red blood cells (43).

In 1953, Amos (3) introduced a leukocyte agglutination technique for the detection of isoantibodies in mice. However, this technique was very laborious. The cytotoxic isoantibody titration introduced by Gorer and O'Gorman (46) in 1956 provided a method whereby H-2 components could be investigated on target tissues composed of nucleated cells. The H-2 isoantibodies were shown to be active in vitro when exogenous complement was added. The cytotoxic isoantibody titration is not plagued by a long prozone such as is often present in the human serum-dextran method. The cytotoxic isoantibody and the human serum-dextran techniques remain useful tools for the detection of isoantibodies and the investigations of genetic configurations. The use of these two methods has enabled Amos, Gorer and Mikulska (9),

Gorer (43,44,48,49) and Amos (6) to elicit some of the histocompatibility configurations of the mouse.

Many attempts have been made to increase the sensitivity of the cytotoxic isoantibody titration. In 1962, Boyse et al. (19) using very dilute tumor cell suspensions claimed to have demonstrated complete sensitivity of target tissue cells to the action of cytotoxic isoantibody and complement. Terasaki (113) found that the sensitivity of the titration for weak antisera could be markedly increased by removing the isoantiserum following incubation with the target cells and before the addition of complement. In 1964, Terasaki and Rich (114) used a Coulter Counter to determine the titer of cytotoxic isoantisera. The upper and lower discriminators were set to encompass the predetermined mean lymphocyte size. Cells which were killed by the action of cytotoxic isoantiserum and complement were swollen or lysed and thus were not counted.

It was once thought that there were two types of antigens present on tissue cells. The H antigens were present on cell membranes, stable to lyophilization, induced isoantibody production and produced enhancement of graft survival rather than transplantation immunity. The T antigens were associated with nucleated cells, labile to lyophilization and induced transplantation immunity without inducing hemagglutinin formation (75). These two antigens are now thought to be identical (20,107), as it has been shown that isoantibodies are regularly formed at the time of homograft rejection (7,19,20,43,80,124,125). However, these isoantibodies often do not affect, in vitro, the tissue

cells which stimulated their production (5).

The role of cytotoxic isoantibodies in allograft rejection is a subject of controversy. It is generally accepted that allograft rejection is an immunological phenomenon in the category of actively acquired immunity. But it is not firmly established whether the graft is destroyed by host cells, isoantibody or a combination of the two mechanisms. Evidence in favor of rejection by host cells has included: (1) Grafts placed over semi-permeable membranes or in diffusion chambers survive in both immune and non-immune hosts, provided that the pores of the membrane or chamber are not large enough to permit the passage of cells (2,5). (2) Not all tissues are demonstrably sensitive to cytotoxic isoantibodies (5,43,60,78,80,91,113,124). Leukemias and lymphomas are almost completely sensitive whereas skin and sarcomas are comparatively resistant (8). (3) It has not been possible to demonstrate consistently isoantibodies after allograft rejection (49,55,77). Evidence that the mechanism of rejection depends solely on the action of isoantibody has included: (1) Immunity to leukemia can be transferred to non-immune hosts by small volumes of isoantiserum administered up to 7 days before tumor implantation (5). (2) Isoantibodies are regularly formed at the time of allograft rejection (7,19,20,43,80,124, 125) and often before morphological signs of allograft rejection (49). However, these isoantibodies may be incomplete and thus only demonstrable in vitro (7,19,49,80), by inhibition or augmentation of a strongly active isoantibody directed against the same isoantigens, and often they do not damage, in vitro, the cells against which they were formed (5). (3) Leukemia and

lymphoma grafts can readily be neutralized with isoantiserum. Many workers have proposed that allografts are destroyed by cell-bound cytotoxins on the basis of the following evidence: (1) Small non-vascularized grafts will survive indefinitely in non-immune hosts, but not in immune hosts (2). (2) Transfer of large quantities of immune cells to non-immune animals will confer a state of immunity and while humoral antibodies can readily be separated from cells, it is difficult to obtain cells free of antibody (5). (3) Allotransplantation produces cell-bound substances which will destroy a variety of tissues (80,123). The highest concentrations of these substances are found on the cells of the spleen and lymph nodes. (4) Treatment of cultures of L cells with sensitized lymphocytes results in aggregation of the lymphocytes around the L cells. Both lymphocytes and L cells show signs of degeneration (99,100). Similar results have been obtained with fibroblasts treated with sensitized spleen cells (111). However, normal cells or homogenates of sensitized cells are ineffective (100,111). (5) Phagocytosis of a variety of nucleated cells depends on the presence of specific isoantibody. The phagocytosis is specific for the cells against which the antiserum is directed. The majority of the phagocytized cells were viable at the time of ingestion. Phagocytosis will occur with unwashed macrophages from an immune donor or with non-immune macrophages suspended in antiserum (14). However, it is difficult to see why large quantities of antiserum alone are ineffective in graft rejection while the same antibody bound to cells is effective. Amos (5) has proposed another possible mechanism of graft rejection. It is suggested that the host

cells become coated with antigens from the graft and complement in the presence of antibody lyses the coated cells. The enzymes released from the lysed cells lyse nearby graft cells which then perpetuate the reaction (49). The actual mechanism of allograft rejection is still unsettled. However, cytotoxic isoantibodies possibly play an important role.

Cytotoxic isoantibodies are implicated in immunological enhancement. Enhancement of a tumor consists of the successful establishment of a tumor allograft and its progressive growth as a consequence of contact between the tumor and specific isoantibody. The isoantibody may be present by virtue of either active or passive immunization (30,43,61). Three possible mechanisms of enhancement have been proposed: (1) The isoantibody may react with the tumor isoantigens, preventing them from evoking host immunity (30,43). (2) The isoantibody may exert a selective pressure by which cells compatible with the foreign host are selected (30). (3) The tumor cells may undergo a "physiological" change which allows them to survive (30). Although the second possibility seems the most likely (30), Feldman and Yaffe (32) conducted a series of experiments which may indicate the third mechanism. They found that a C57Bl-specific sarcoma transplanted to an irradiated C3H recipient would proliferate and subsequently lose its strain specificity. It was suggested that the loss of strain specificity was not a result of a gradual selection of compatible cells from pre-existing cell variants, but an immunogenetic transformation by the host's environment. This transformation proved to be reversible, for strain specificity was restored following

transplantation back to the strain of origin of the tumor. It was thought that a decrease in antigen emission from the tumor had accompanied its transplantation in the foreign host. The possibility arises from this that cytotoxic isoantibodies may produce enhancement by influencing antigen emission from graft cells.

II. MATERIALS

A. Animals

Four strains of mice were used, C3H, C57L, Strong A, and LAF_1. The C3H and C57L mice were raised in this laboratory by strict brother and sister mating. The original C3H mice (78th generation) were obtained from the Department of Cancer Research, University of Saskatchewan, in April, 1961, and the original C57L mice from Roscoe B. Jackson Memorial Laboratories, Bar Harbour, Maine, in May, 1960. Strong A and LAF_1 mice were obtained in bi-monthly consignments from Roscoe B. Jackson Memorial Laboratories. The mice were housed in stainless steel, glass, plastic or fiberglass cages with wood shaving nesting material. Vit-a-mite cubes[a] and water were supplied ad libitum.

The histocompatibility genetic constitution of the four strains of mice is shown in Table I. The histocompatibility genetic pattern was taken from a table supplied by Roscoe B. Jackson Memorial Laboratories.

As the C3H and C57L mice had been maintained at this laboratory for a few generations, skin grafts were done to insure that there had been no major deviations in histocompatibility loci. Several C3H mice were selected at random through the colony and exchange skin grafts performed. The C57L mice

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[a]Northwest Feed Mills, Edmonton.

Table I

Histocompatibility Genetic Constitution of Mice

Strain	H-2 Genotype	Antigenic Components
C3H	$H\text{-}2^{k}$	AA CC $D^{k}D^{k}$ EE -- HH -- -- KK -- -- -- -- -- -- YY ZZ
C57L	$H\text{-}2^{b}$	-- -- $D^{b}D^{b}$ EE FF -- -- -- $K^{b}K^{b}$ -- NN -- -- -- VV -- ZZ
Strong A	$H\text{-}2^{a}$	AA CC DD EE FF HH -- JJ KK MM NN -- -- -- -- YY ZZ
LAF_{1}	$H\text{-}2^{a}xH\text{-}2^{b}$	A- C- DD^{b} EE FF H- -- J- KK^{b} M- NN -- -- -- V- Y- ZZ

were similarly grafted. All of the grafts were accepted permanently. As a further check, several C57L mice were grafted with skin from LAF_1 mice. These grafts were also permanently accepted.

B. Glassware

All glassware which was to have prolonged contact with live spleen cells was cleaned with OrganiSol[a] and siliconized with Siliclad.[b] Table II illustrates the effectiveness of chemical cleanliness, paraffin coating and siliconization in reducing non-specific cell death. These tests utilized 0.1 ml. of normal C3H spleen cells (2×10^6), 0.1 ml. of undiluted C3H serum and 0.1 ml. of 1:8 guinea pig complement.[c]

C. Stains

Both eosin Y and lissamine green[d] have been used to stain dead cells selectively in cytotoxic isoantibody titrations. Lissamine green was the dye of choice for the color was easier to observe than that of eosin Y. Holmberg (56) stated that lissamine green was non-toxic and penetrates instantaneously into the cytoplasm and nucleus of cells showing the cytological changes characteristic of irreversible cell injury. The fresh dye solution was made up every week, for the color tends to deteriorate. Fixed preparations (page 22) were stained with Trypan blue,[e] as lissamine green faded from the stained cells

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[a] J. R. Gilbert Ltd., Toronto 9, Ontario.
[b] Clay-Adams, Inc., New York 10, New York.
[c] Connaught Medical Research Laboratories, Toronto, Ontario.
[d] Gurr's, Esbe Laboratory Supplies, Toronto, Ontario.
[e] Allied Chemical Corporation, New York 6, New York.

Table II

Comparison of Three Methods of Treating Test Tubes to Reduce Non-Specific Cell Death

Treatment	Percentage of Stained Cells								
Chemically Clean	9.9	4.4	0.7	3.1	3.1	7.2	0.6	10.4	9.2
Paraffin Coated	36.0	42.2	12.9	15.7	42.0	6.0	8.0	3.9	12.9
Siliconized	1.9	1.0	1.1	0.8	0.7	0.9	1.2	2.1	0.9

in 24 hours. Trypan blue precipitates markedly in saline and was therefore made up in sterile distilled water to a concentration of 5%. Immediately before use, the aqueous solution of Trypan blue was diluted 1:10 with sterile 1% saline and filtered.

III. METHODS

A. Preparation of Cytotoxic Antisera

Isoantiserum used in these experiments was produced by intraperitoneal immunization with lyophilized or fresh allogenic tissue cells emulsified in complete Freund adjuvant.[a] The most potent of the histocompatibility antigens are the complex H-2 antigens. The H-2 antigens are widely distributed amongst the normal tissues of the mouse (4,43,93,117), and although the various organs of the mouse differ widely in their isoantigen content, a given organ has a constant content under normal conditions (11). The spleen, having a good immunizing capacity (104), was used for immunization. The H-2 isoantigens are primarily cytoplasmic in location and appear to be associated with surface membranes (11). Cytotoxic isoantibodies exert their effects on the surface membranes (62,68,80,82,91,94) and are therefore thought to be primarily directed against the H-2 isoantigens (22,108).

The use of complete Freund adjuvant with an antigen enhances the production of antibody (53,58) and produces a marked histiocytic response. Elevated antibody titers and prolonged response were probably due to the retention and gradual release of antigens from the newly proliferated

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[a]Bacto Complete Adjuvant, Difco Laboratories, Detroit, Mich.

reticulo-endothelial cells and histiocytes (85). The adjuvant provides a depot for the persistence of antigens (21). Adjuvant also stimulates the development and multiplication of antibody producing cells, and effectuates a wide distribution of antigens through the tissue, making the antigens more accessible to antibody-forming sites (22).

1. Fresh Spleen Cells

Generally, isoantiserum was produced as follows: A normal donor mouse was sacrificed by ether[a] inhalation and decapitation. The spleen was removed aseptically and teased apart in a 0.2 ml. of phosphate buffered saline[b] (PBS) (pH 7.2) solution with the edge of a sterile 50 gauge stainless steel screen. The resulting spleen suspension was then emulsified with 0.8 ml. of complete Freund adjuvant. Each of the mice to be immunized received an intraperitoneal injection of 0.1 ml. (2×10^7 cells per ml.) of freshly prepared spleen emulsion on days 0, 7, 52, 87, 97 and 107.

Blood, collected from the tail veins of immunized mice, was allowed to clot at room temperature and the serum was removed by centrifugation at 1725 x g. Sera from individual mice of the same experimental group and day were pooled, and if not used immediately were stored at -20°C. Cytotoxic titrations were carried out on sera collected 25, 82, 92, 102 and 112 days

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[a] E. R. Squibb and Sons, Ltd., Montreal, Canada.

[b] Solution A: 80.0 grams NaCl, 2.0 grams KCl, 1.0 grams $CaCl_2$, 1.0 grams $MgCl_2 \cdot 6H_2O$ diluted to 1 liter. Solution B: 11.5 grams $Na_2HPO_4 \cdot 12H_2O$, 2.0 grams KH_2PO_4 diluted to 1 liter. Working Solution: 100 ml. of A are added to 800 ml. of double distilled water, then 100 cc. of B are added.

after the initial immunizing injection.

2. Lyophilized Spleen Cells

Lyophilized spleen cells were prepared as follows: A normal C57L mouse (male or female) was sacrificed by ether inhalation and decapitation. The spleen was removed aseptically and teased apart in 0.2 ml. of PBS with a sterile 50 gauge stainless steel screen. The spleen suspension was then transferred to a sterile ampule. Freeze-drying was performed on an Edwards Speedi Vac (Model 5PS) high vacuum centrifugal freeze dry machine. The lyophilized spleen suspension was then transferred to a sterile mortar and resuspended in 0.2 ml. of sterile distilled water with a sterile pestle. When the resulting suspension was examined for live and intact cells, none were found. The reconstituted spleen suspension was then emulsified with 0.8 ml. of complete Freund adjuvant and 0.1 ml. injected intraperitoneally into C3H male recipients.

Immunization and bleeding schedules were the same as those for fresh spleen cells described on the previous page.

B. Demonstration of Isoantibodies

1. Cytotoxic Titration

The cytotoxic titration was a modification of the Gorer and O'Gorman method (46). By this method, cells which were not permeable to the dye were termed resistant to the antibody.

Unless otherwise stated, all spleen cell suspensions were prepared in the following manner: a normal mouse was sacrificed by decapitation with a large pair of scissors while lightly anaesthetized with ether. This method of sacrifice

reduced the amount of blood in the tissues and provided normal serum when needed for controls. The spleen was removed aseptically and suspended with a sterile 50 gauge stainless steel screen in 2.0 ml. of PBS in a sterile petri dish. This spleen suspension was then diluted 1:10 with PBS in a clean siliconized test tube. The resulting spleen suspension, which contained approximately 2×10^7 cells per ml. was then stored at 4°C. for one hour before use in the titration. Cold storage reduced the number of stained cells detected in the control. This was probably due to lysis of cells which were injured during the preparation of the spleen cell suspension (104). The decreased numbers of cells detected in the spleen cell preparations was not due to increased "sticking" of cells to the surface of the test tube. A C3H spleen cell suspension was prepared as described previously. The spleen cells were allowed to stand for one hour at 4°C., after which the tube was drained. To the test tube was added 0.3 ml. of 0.25% Trypsin.[a] After rotating gently for two minutes, a sample was examined for spleen cells; from 16 to 33 cells were found in a standard area of a hemocytometer. This number of cells was well within the expected number of cells that would be left on the inside of the test tube. Table III illustrates the drop in numbers of spleen cells in such a suspension. There was a rapid drop in the number of cells counted from the time the suspensions were made (0 time) to 30 minutes. After 30 minutes of standing in the cold, the

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[a]Difco Laboratories, Detroit, Michigan; proportion 1:250.

Table III

Number of C3H Spleen Cells Counted in Standard Area of Hemocytometer

	Time at 4 °C. (minutes)			
Count	0	30	60	90
1	791	509	416	427
2	506	348	294	-
3	399	210	197	-
4	536	356	-	-
5	546	368	-	-
6	557	368	-	-

drop in number of spleen cells counted was more gradual. All spleen cell suspensions were therefore stored at 4°C. for one hour before use, and it is assumed that any cells which were injured or killed by the trauma of suspension, had disintegrated.

The concentration of spleen cells was found to influence greatly the results of antibody titrations. Concentrations of 10^7 cells per 0.1 ml. decreased the titer, and such titrations were also difficult to count due to the large numbers of cells. Preparations of spleen cells containing more than 8×10^6 cells per 0.1 ml. were often highly anti-complementary (12). Concentrations of 10^5 or 10^4 cells per 0.1 ml. were very difficult to count unless the suspensions were concentrated before counting. Also, with such dilute suspension of cells, non-specific killing effects of dilution were present.

The non-specific killing effects of dilution were present even as the cytotoxic titration was normally done. As normal serum was diluted out, the percentage of stained cells increased (Figure 1) to a dilution of about 1:8 normal serum, where a plateau was reached for the remaining dilutions at about 30%. It seemed that dilution of the cells increased cell death, probably due to a loss of the protective effect of serum, and was a non-specific result of dilution itself. The control for most of the dye exclusion titrations was undiluted normal serum. When serial doubling dilutions of normal serum were prepared and the percentage of dead cells in each dilution used to correct the corresponding dilution of antiserum, the $\log_2$ titer is reduced by one $\log_2$ unit (Figure 2). It is of prime importance that the number

Figure 1. Effect of various dilutions of normal C57L serum and 1:8 guinea-pig complement on the viability of C57L cells after incubation at 37°C. for 30 minutes.

Figure 2. Titration of C3H anti C57L serum against C57L spleen cells in the presence of 1:8 complement. Percent relative mortality calculated from a single undiluted C57L serum control (●) and from a complete series of C57L serum dilution controls (o).

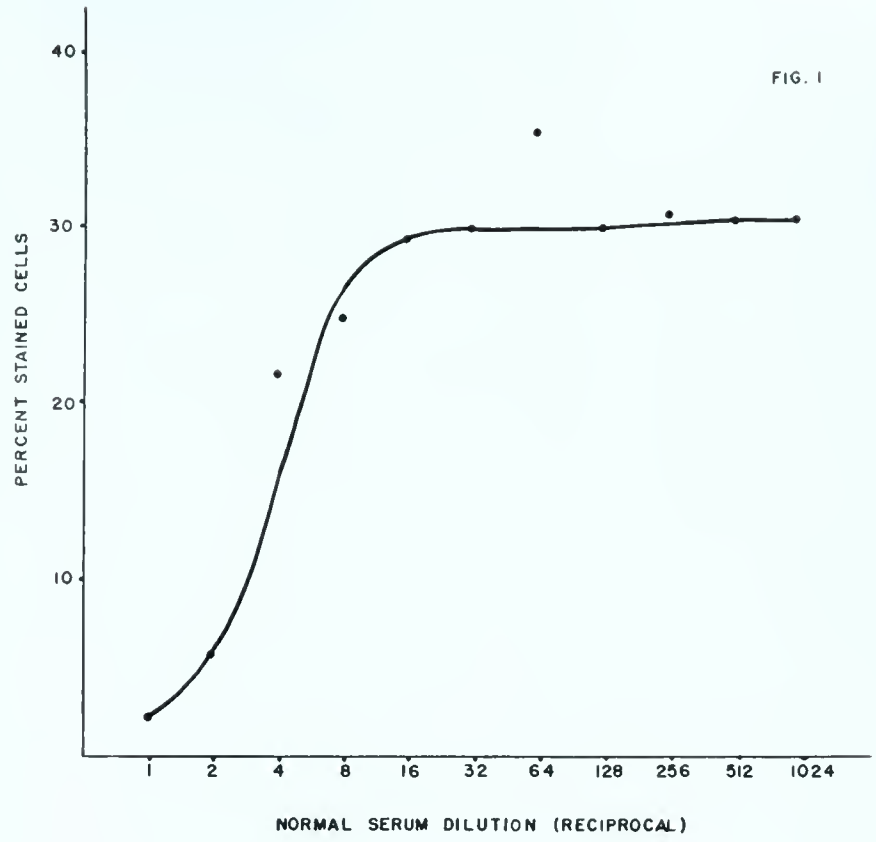
40
30
20
10
PERCENT STAINED CELLS
1
2
4
8
16
32
64
128
256
512
1024
NORMAL SERUM DILUTION (RECIPROCAL)

FIG. 1

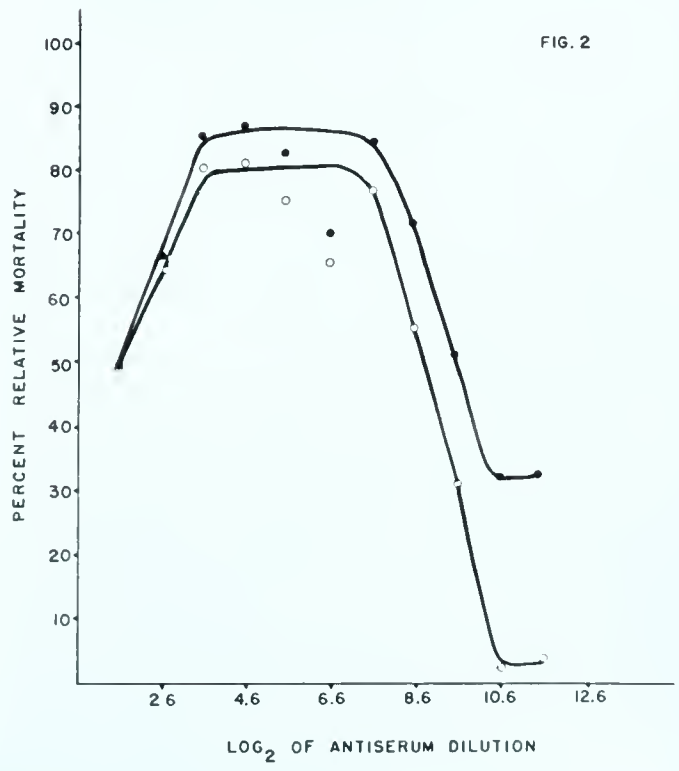
100
90
80
70
60
50
40
30
20
10
PERCENT RELATIVE MORTALITY
2.6
4.6
6.6
8.6
10.6
12.6
LOG2 OF ANTISERUM DILUTION

FIG. 2

of cells be consistent if comparisons are to be made from test to test.

The test system consisted of 0.1 ml. of the appropriate antiserum doubly diluted in PBS from 1:1 to approximately 1:1024. To each tube of antiserum dilution were then added 0.1 ml. of a 1:8 dilution of reconstituted lyophilized guinea-pig complement in PBS and 0.1 ml. of the appropriate spleen cell suspension (2×10^7 cells per ml.). A control containing normal serum from the spleen cell donor was also set up. All tubes were then corked and incubated in a water bath at 37°C. for 30 minutes. After incubation, 0.3 ml. of 1.5% lissamine green in PBS was added to each tube. The suspensions were counted for proportion of stained cells in a hemocytometer, counting a total of 200 to 400 cells per tube. The percent relative mortality was calculated from the formula: (126)

$$100 \left[1 - \frac{100 - \text{test \% dead cells}}{100 - \text{control \% dead cells}} \right]$$

Titers were expressed as the $\log_2$ of the antiserum concentration which resulted in 50% relative mortality.

Mouse complement is usually quite inactive *in vitro* (5, 10,43,80,98,101,122,124). Complement from another species must be added if cytotoxicity of mouse isoantiserum is to be demonstrated (15,51,52,60,80,91). Complement concentration is very important and will be dealt with in a later section. It suffices to say at this point that 1:8 complement provides an excess of active complement.

Cytotoxic titrations done at different times with different spleen cell suspensions were compared to test the assumption that all spleen cells from mature mice of one strain were equally susceptible to a given isoantiserum. That this was so is illustrated by the following experiment. Five C3H mice (3 male and 2 female) approximately 3 months old, were sacrificed and suspensions of each spleen were made as described previously. Each spleen suspension was titrated against C57L anti C3H serum with complement according to our standard technique. The 50% end point $\log_2$ titers of the five spleens tested were in agreement (Table IV).

2. Fixed Cell Preparations

Fixed spleen cell preparations were made as follows: 0.6 ml. of 1:8 guinea pig complement and 0.6 ml. of appropriate target spleen cells (6×10^6) were added to 0.6 ml. of each antiserum dilution in siliconized test tubes. The tubes were incubated at 37°C. in a water bath for 30 minutes. To each tube was added 0.6 ml. of 0.5% Trypan blue in saline. All tubes were then centrifuged at 769 x g for 5 minutes. The supernatant was removed and the sediment transferred to 1 ml. siliconized centrifuge tubes. The sediment was washed three times with 0.6 ml. of saline, centrifuging at 769 x g for 5 minutes each time. Coverslip films made from the sediment were fixed in ethanol, cleared in xylol and mounted with permount. The slides were examined by phase microscopy under oil immersion (x1000). Controls with normal serum were prepared similarly, using the same spleen suspensions.

Table IV

Percentage of Stained Cells Obtained with Five Different C3H Spleen Cell Suspensions Treated with C57L anti C3H Serum

Spleen Suspension	Antiserum (C57L anti C3H) Dilution*			Normal C3H Serum*	50% Relative Mortality Titer (log_2)
	1:2	1:4	1:8		
1	55.2**	35.4	10.7	4.5	2.9
2	56.2	40.8	30.5	3.8	3.0
3	58.2	38.2	25.7	3.9	3.0
4	52.4	42.5	34.1	4.3	2.9
5	59.7	46.5	28.8	5.4	3.1

*1:8 complement used throughout.

**200-400 cells counted per tube.

3. Hemagglutination Technique

(a) Human serum-dextran method

Hemagglutination titrations were done by a modification of the Gorer and Mikulska (47) human serum-dextran method.

A normal mouse was sacrificed by ether inhalation and decapitation. Blood was collected into 0.4 ml. sterile acid citrate dextrose[a] in a sterile centrifuge tube. The cells were washed three times in sterile normal saline, centrifuging at 1725 x g after each washing. The spleen, liver and kidney were removed aseptically and minced with sterile scissors in sterile normal saline. The minced tissues were washed three times in sterile saline and finally centrifuged at 1725 x g for 15 minutes. Normal human serum collected from a fasting, healthy Caucasian male, 23 years of age, was inactivated at 56°C. in a water bath for 30 minutes and then added to washed, minced normal mouse tissue. The tissue and human serum were allowed to stand for 2 hours at 37°C., after which the serum was removed by centrifugation at 1725 x g for 15 minutes. The adsorped human serum was then reinactivated in a 56°C. water bath for 10 minutes. The normal mouse red blood cells were then made into a 2% solution in the adsorped inactivated 1:2 human serum in saline.

In a plexiglass tray, 0.1 ml. of the appropriate antiserum which had been inactivated in a 56°C. water bath for 30 minutes was then doubly diluted in 0.1 ml. of 1:3 dextran[b] in

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[a] Sodium citrate 1.32 grams, citric acid 0.48 grams, dextrose 1.47 grams, diluted to 100 milliliters with distilled water.

[b] Intradex, Glaxo Laboratories, Ltd., Greenford, England.

saline. To each well was then added 0.1 ml. of the 2% mouse red cell suspension in human serum. The tray was shaken and incubated at 37°C. for 2 hours. After incubation, the tray was again shaken and the dilutions read for macroscopic agglutination. The titer was established as the last well (dilution) that gave a positive agglutination. Doubtful agglutination was considered negative. Controls containing 1:3 dextran-saline and 2% mouse red cells-human serum were included for each strain of mouse cells tested.

Normal human serum was collected from a fasting volunteer. It was found that visible cloudiness in the serum due to lipid completely inhibited agglutination.

(b) Saline method

Saline agglutination was also tested for each antiserum. The antiserum was inactivated in a 56°C. water bath for 30 minutes. The inactivated antiserum (0.1 ml.) was doubly diluted in 0.1 ml. of normal saline. To each well were added 0.1 ml. of 2% mouse red cells in normal saline. Controls of 0.1 ml. 2% mouse red cells in saline and 0.1 ml. of normal saline were included. Incubation and reading for agglutination were carried out as described above.

4. Complement Fixation Technique

In this method, the modified cytotoxic isoantibody titration of Gorer and O'Gorman (46) was done as usual. However, a hemolytic system was added and the presence of isoantibody was determined by complement utilization rather than by dye exclusion. This system was similar to one utilized by Winn (124).

All serum was inactivated at 56°C. for 30 minutes before use.

Test: Doubling dilutions of 0.1 ml. of antiserum were made in 0.1 ml. of PBS in siliconized test tubes. To each tube were added 0.1 ml. of 1:32 guinea pig complement and 0.1 ml. of the target spleen cells.

Antiserum control: Doubling dilutions of 0.1 ml. of inactivated antiserum were made in 0.1 ml. of PBS. To each tube were added 0.1 ml. of 1:32 complement (C') and 0.1 ml. of PBS.

Serum control: 0.1 ml. of antiserum was doubly diluted in 0.1 ml. of PBS. To each tube was then added 0.2 ml. of PBS.

Cell control: 0.1 ml. of target spleen cells were pipetted into five tubes. To each of these tubes were added 0,1 ml. of 1:32 C' and 0.1 ml. of PBS.

Hemolysin control: 0.1 ml. of 1:32 C' was pipetted into five test tubes. To each of these tubes was added 0.2 ml. of PBS.

The test, antiserum control, serum control, cell control, and hemolysin control tubes were corked and incubated for 30 minutes in a 37°C. water bath. After incubation, 0.1 ml. of 1:1000 rabbit anti sheep red blood cell hemolysin[a] in Kolmer saline[b] and 0.1 ml. of 2% sheep red blood cells in Kolmer saline were added to each tube. The series were then incubated for a further 30 minutes. At the end of the second incubation, all tubes were centrifuged at 1725 x g for 15 minutes. The

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[a] Department of National Health and Welfare, Ottawa, Canada.
[b] 0.85%: 17 grams NaCl, 2 liters double distilled water, 2.0 ml. 10% $MgSO_4$, filter before use.

supernatant was removed and 0.35 ml. of each supernatant was added to 2.0 ml. of Drabkin's solution.[a] After standing for 10 minutes, the optical density at 540 mμ was determined using a Coleman Jr. Spectrophotometer and a Drabkin solution blank. In addition, serum color was determined. In 0.1 ml. of PBS, 0.1 ml. of antiserum was serially doubly diluted. To each antiserum dilution, 0.4 ml. of PBS was added. From each tube 0.35 ml. was added to 2.0 ml. of Drabkin's solution and the optical density determined as above.

The optical density readings of serum color were subtracted from all tests which contained serum. The optical density of the spleen cell control was taken as the 100% hemolysis for all tubes which contained spleen cells, while the hemolysin control served as 100% hemolysis for all tests which did not contain spleen cells. The percent hemolysis of each dilution of the antiserum control was then subtracted from 100, and the resulting figure added to the corresponding dilution of antiserum in the test. This gave a percent hemolysis which had been corrected for color and anti-complementary effect of antiserum.

The hemolysin used was in excess of that needed, for with 1:32 C' the hemolysin titer was usually found between 1:5000 and 1:6000. This concentration of hemolysin, however, ensured complete hemolysis whenever complement was in excess.

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[a] $NaHCO_3$, 1.0 grams; KCN, 50 milligrams; $K_3Fe(CN)_6$, 200 milligrams and diluted to 1 liter with distilled water.

IV. CHARACTERISTICS OF CYTOTOXIC ANTISERA

A. Shape of the Titration Curve

Figure 3 illustrates the shape of the curve of a typical cytotoxic isoantibody titration by the modified Gorer and O'Gorman method (46). The percent relative mortality was lower at high antiserum concentrations, rose to a plateau, then fell rapidly between 70 and 40% and leveled off around 35%. Variations in the estimate of relative mortality of 10 to 20% would not change the 50% $\log_2$ titer by more than one unit. Therefore, the 50% relative mortality end point would seem to be the most reproducible method of estimating the $\log_2$ titer. It was noted that 100% relative mortality was never reached.

The decreased mortality at high antiserum concentrations may be due in part to the anti-complementary action of mouse serum and in part to an isoantibody excess. It had also been found that undiluted mouse sera was protective to spleen cells (111) and this may account for the increased viability.

Gorer and O'Gorman (46), Bennett et al. (14), Cann and Herzenberg (22), and Reif and Norris (35) have also noted that the percentage of stained cells never reached 100%. They attributed this phenomenon to a portion of resistant cells. However, Boyse et al. (19) had stated that 100% mortality could be obtained if very dilute suspensions of target cells were

Figure 3. Typical cytotoxic isoantibody titration curve. C57L anti C3H serum titrated against C3H spleen cells in the presence of 1:8 complement. Broken line illustrates method of determining 50% end point.

Figure 4. Percentage of stained cells (----) and percent relative mortality (———) of 1:4 (●), 1:64 (o), and 1:1024 (♦) dilutions of C3H anti C57L anti C57L serum plotted against four C57L spleen cell concentrations.

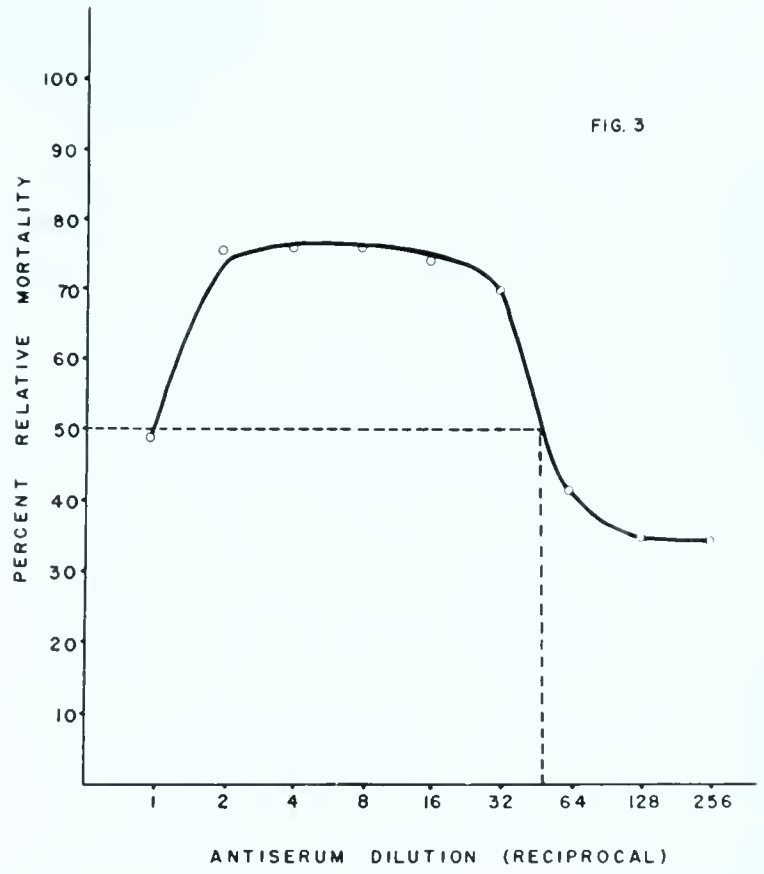

100
90
80
70
60
50
40
30
20
10
PERCENT RELATIVE MORTALITY
1
2
4
8
16
32
64
128
256
ANTISERUM DILUTION (RECIPROCAL)

FIG. 3

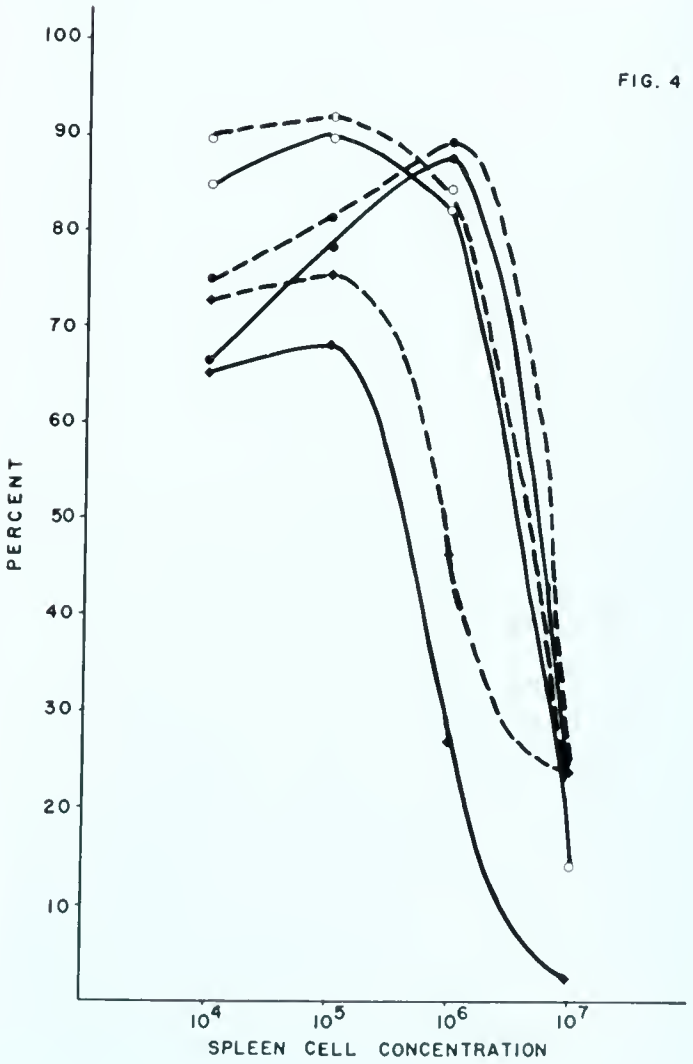

100
90
80
70
60
50
40
30
20
10
PERCENT
10^4
10^5
10^6
10^7
SPLEEN CELL CONCENTRATION

FIG. 4

used. Figure 4 illustrates the percent relative mortality obtained with three C3H anti C57L serum dilutions titrated with four different concentrations of target C57L spleen cells and 1:16 guinea pig complement. These titrations indicated that a very dilute cell suspension decreased the percentage of stained cells detected with a low dilution of antiserum, but increased the numbers of dead cells with a high dilution of antiserum. The percent relative mortality of a moderate dilution of antiserum (1:64) remained fairly constant about the range of 10^4 to 10^6 cells. The cytotoxic activity of the serum was markedly reduced with all three antiserum dilutions when the serum was titrated with 10^7 spleen cells. However, 100% stained cells was not obtained with any of the cell dilutions. There appeared to be a small part of the spleen cell population which was resistant to the action of cytotoxic isoantibody. Cann and Herzenberg (22) have selectively cultured these resistant cells using tissue cultures. Two to five months later, these clones showed varying resistance from complete to partial. The variant cells appeared larger than the cells of the parent population. They attributed resistance to cytotoxic isoantibodies to the larger size in that it altered the spatial relationship for optimum binding of isoantibody and complement. Fixed smear preparations were prepared as described on page 22 and the unstained cells examined. These cells were divided, on the basis of size, into three groups: large, medium and small (Figure 5). Each of these groups were subdivided on the basis of amount of cytoplasm. Occasionally, a large cell with a lobulated nucleus was seen. The medium-sized cells with a small rim of cytoplasm (MS) and the small cells with

Figure 5. Large (L), medium (M), and small (S) cells present in a spleen cell population. C57L spleen cells treated with C3H anti C57L serum. Arrows indicate stained cells. Trypan blue stain, phase microscopy, enlarged from x1000 mag.


5
s
m

small rims of cytoplasm (SS) varied greatly with the antiserum concentration, while the other cell types remained fairly constant in number. Table V illustrates the changes in cell population with increasing antiserum concentrations.

The medium-sized cells with small rim of cytoplasm appeared to be the most sensitive to cytotoxic isoantibody, while the small-sized cells with small rim of cytoplasm appeared to be resistant to the action of cytotoxic antibody. It would appear then that 100% mortality was never achieved as a portion of the target cells, probably the small cells, were resistant to the cytotoxic action of antiserum.

B. Morphological Effects of Cytotoxic Antiserum on Target Cells

When in the presence of active complement, cytotoxic isoantibodies react with the surface antigens of the cell membranes (62,68,80,82,91). However, specific isoantiserum will not produce the structural changes alone (52,60), for cells may be grown in tissue culture in the presence of a significant concentration of isoantibody (52). Active complement must be present for the isoantibody-isoantigen reaction to produce the characteristic structural changes (15,51,52,60,80,91). In 1960, Green and Goldberg (52) utilized the electron microscope in a study of the surface membranes of cells which had been treated with heterologous antibody. They found that the cellular membranes were altered by a series of invaginations or projection when the cells were treated with antibody alone. At the addition of active complement, the cells became swollen. This swelling process began with numerous peripheral blebs which fused to form a uniform "balloon" around the cell. The membrane-bound compartment also

Table V

Percentages of Unstained Medium and Small Cells with Scant Cytoplasm Observed in Fixed Spleen Cell Preparations

Antiserum	No. Unstained Cells Counted Per Slide	Antiserum Dilutions												1:16 Normal Serum	
		1:16		1:64		1:256		1:1024		1:4096		1:16384			
		SS	MS	SS	MS	SS	MS	SS	MS	SS	MS	SS	MS	SS	MS
A anti C57L	650	41.6	20.6	-	-	-	-	-	-	-	-	-	-	7.1	57.5
C57L anti A	500	94.2	3.2	91.8	4.8	79.2	16.8	62.2	34.8	51.8	45.2	29.6	66.0	9.2	84.4
C3H anti C57L	500	69.6	25.4	58.8	36.2	50.2	42.6	50.2	42.2	34.4	57.4	22.8	67.6	8.4	81.0

became swollen. The cells were not lysed by the reaction; however, the membranes became extremely fragile and were very liable to mechanical disruption. These results confirm those observed by others using ordinary light microscopy (15,37,60,62,68,76,78,80,91,112) and time lapse cinematography (110).

Cytotoxic isoantibody in the presence of active complement exerted its action by interference with the permeability of the surface membrane, the metabolism of the cell, or the sodium-potassium pump (76). There was a rapid equilibrium of small molecules between the cell interior and the medium. Potassium ions were lost more rapidly than the normal leak and as a consequence, sodium ions passed into the cell (52,76). Green et al. (51) found that the cells lost 90% of their intracellular potassium ions, two-thirds of their free amino acids and ribonucleotides, three-quarters of their ribonucleic acid, and 30 to 60% of their cellular protein. The particles lost were intact and could be recovered by differential centrifugation. Immune cytolysis has, therefore, been defined as the leakage of intracellular macromolecules through a stretched but morphologically intact cell membrane (52). Any condition which leads to the equilibrium of ions between the cell and the medium will produce an unbalanced colloid osmotic pressure, and water will flow into the cell. Cytotoxic isoantibody in the presence of active complement is capable of so altering this equilibrium.

C. Specificity of Cytotoxic Antisera

The method of inducing cytotoxic isoantibody production was a rigorous immunization program in which the recipient mice eventually received six intraperitoneal injections of donor

spleen cells and adjuvant. The possibility that this intensive immunization may have produced an antibody which was not specific for the donor tissues was investigated. A C3H anti C57L serum was titrated against C57L and C3H spleen cells according to the standard method described previously (page 21). This antiserum was found to be highly cytotoxic to C57L cells ($\log_2$ titer of 8.9) but was not lethal to C3H cells. Similar results were obtained when C57L anti C3H serum was titrated against C3H and C57L spleen cells.

The antisera produced then, were specific for the antigens absent in the recipient strain but present in the donor strain. These antisera will cross react with other strains which have antigens common to those of the donor strains. Table VI illustrates some of these cross reactions. An anti C57L serum appeared to be directed against alleles M, J and D. C3H anti A appeared to be mainly directed against D, J and M, though some cross reaction occurred with F and N. C3H anti C57L appeared to have at least half of its isoantibody directed against F and N. There appeared to be little correlation between the alleles present and the strength of isoantibody formed against each allele. Probably spatial arrangement of the antigens played an important role in both the production and demonstration of cytotoxic isoantibody. Winn (124) has shown that activity of isoantiserum is not solely dependent on the total amount of isoantibody present, but also on the availability of isoantibody combinations of certain specifications.

Table VI

Cross Reactions with H-2 Antigens on Spleen Cells of Various Strains

Antiserum	Cells			
	A	C57L	C3H	LAF_1
C3H anti C57L				
Foreign antigens	FF NN	D^bD^b FF K^bK^b NN VV	-	D^b- FF K^b- NN V-
$\log_2$ titer	5.8	11.3		7.7
A anti C57L				
Foreign antigens	-	AA CC DD HH JJ KK MM YY	AA CC HH KK YY	A- C- D- HH JJ K- M- Y-
$\log_2$ titer	-	5.8	1	6.2
C3H anti LAF_1				
Foreign antigens	DD FF JJ MM NN	D^bD^b FF K^bK^b NN VV	-	DD^b FF J- K^b- M- NN V-
$\log_2$ titer	8.7	7.2		8.5
C3H anti A				
Foreign antigens	DD FF JJ MM NN	FF NN	-	D- FF J-M- NN
$\log_2$ titer	6.8	2.4		6.4

D. Stability of Cytotoxic Antisera

The cytotoxic and hemagglutinating activity of mouse iso-antiserum was heat stable at 56°C. for 5 to 50 minutes (113).

Some of the sera used in these experiments had been stored from one to twelve months at -20°C. The possibility that storage may affect cytotoxic titer of the antisera was considered, although Winn (114) had stored antiserum for ten months at 4°C. or -20°C. with no change in the titer. A C3H anti LAF_1 serum with a cytotoxic $\log_2$ titer of 9.1 was stored at -20°C. Seven months later, portions of this serum were thawed and incubated for one hour at 4°C., 26°C., 37°C., and 56°C. No cytotoxicity was demonstrable for the sera that had been held for one hour at 4°C. and 26°C., while a cytotoxic $\log_2$ titer of 9.5 was obtained with sera incubated at 37°C. and at 56°C. for one hour. Thus storage of antisera at -20°C. for periods of at least seven months did not affect the titer, provided the sera were "reactivated" by heating at either 37°C. or 56°C. for one hour before titration. Unless otherwise stated, all frozen antisera were "reactivated" at 37°C. in a water bath for one hour.

V. RESPONSES OF MICE TO IMMUNIZING INJECTIONS OF ALLOGENIC SPLEEN CELLS

A. Strain Combinations

The four strains of mice available were compared as to their ability to produce cytotoxic isoantibodies. Groups of donor-recipient pairs were made of all possible combinations of C57L, C3H, Strong A, and LAF_1.

Group	Recipient	Donor
IA	C3H (male)	C57L (male and female)
IB	C57L (female)	C3H (male and female)
IIA	C3H (male)	LAF_1 (male)
IIB	LAF_1 (male)	C3H (male and female)
IIIA	Strong A (male)	LAF_1 (male)
IIIB	C57L (male)	LAF_1 (male)
IVA	Strong A (male)	C3H (male and female)
IVB	C3H (male)	Strong A (male)
VA	Strong A (male)	C57L (male and female)
VB	C57L (male)	Strong A (male)

Immunization and serum collection were carried out as described previously. The results of cytotoxic titration by the modified method of Gorer and O'Gorman (46) are shown in Figures 6 and 7. A single immunizing injection of fresh spleen cells, even when combined with adjuvant, did not produce

Figure 6. Cytotoxic isoantibody titers (50% relative mortality end points) obtained with various strain combinations. Arrows indicate immunizing injections. (● ——) LAF_1 → C3H, (▲——) LAF_1 → C57L, (▲---) Strong A → C3H, (o ---) Strong A → C57L.

Figure 7. Cytotoxic isoantibody titers (50% relative mortality end points) obtained with various strain combinations. Arrows indicate immunizing injections. (● ——) C57L → C3H, (▲——) C57L → Strong A, (o ——) C3H → C57L.

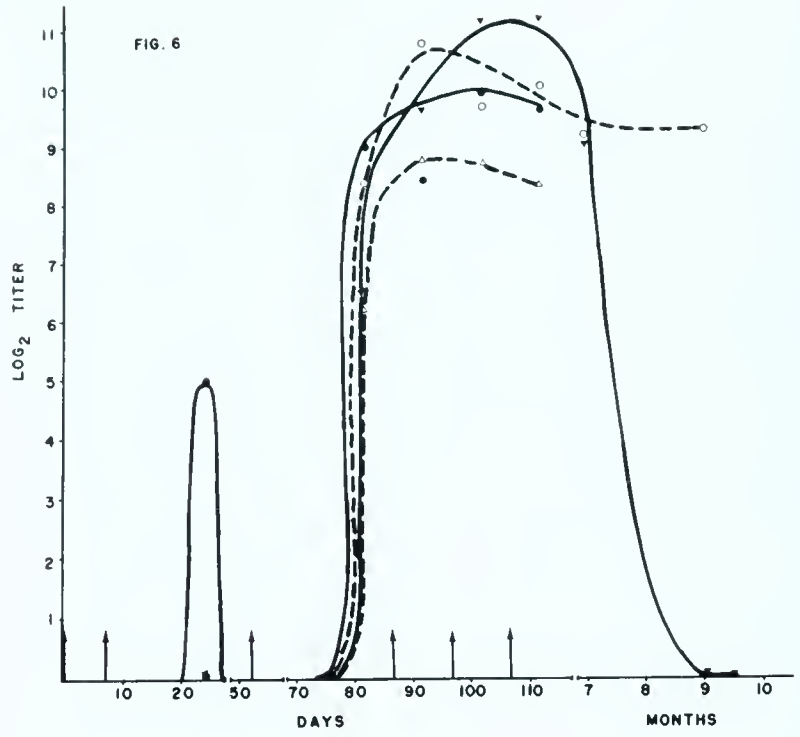


FIG. 6
LOG2 TITER
11
10
9
8
7
6
5
4
3
2
1
10
20
50
70
80
90
100
110
7
8
9
10
DAYS
MONTHS

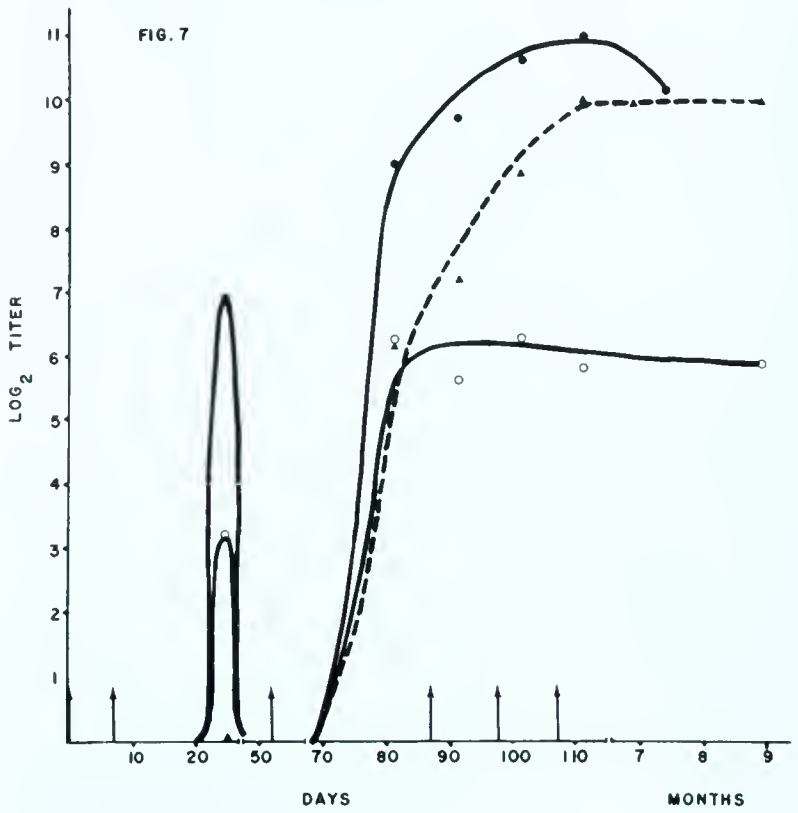


FIG. 7
LOG2 TITER
11
10
9
8
7
6
5
4
3
2
1
10
20
50
70
80
90
100
110
7
8
9
DAYS
MONTHS

detectable antibody. However, after two immunizing injections of fresh spleen cells emulsified in adjuvant, some strain combinations produced a demonstrable cytotoxic titer which reached a peak at about 25 days (primary peak). The isoantibody disappeared rapidly and at about day 30 was no longer demonstrable. A subsequent immunizing injection on day 52 did not result in isoantibody production until day 60 or later. The titer climbed rapidly to reach a peak at day 82 (secondary peak), and booster injections at days 87, 97 and 107 did not further increase the titer appreciably. Antiserum of comparable titer could be obtained from these mice for as long as four to five months after the last immunizing injection.

The shape of the immunization curve was of interest. This type of curve was similar to one obtained by Halbert (53) using rabbit lens protein in Freund adjuvant. Antigen was injected intradermally into rabbits at approximately days 0, 7, 60, 90, 140 and 230. Testing the sera with a hemagglutination technique, he obtained a peak at 1 to 2 weeks followed by a fall at 3 weeks and a subsequent rise.

If the effectiveness of the antigens could be judged simply by the appearance of the immunization curve, it could be said that a strong antigen would be one which would produce both a "primary" and a "secondary" peak; a good antigen, one which produced only a "secondary" peak; and a poor antigen, one which produced no detectable response. If this criterion were to hold, C57L → C3H, LAF_1 → C3H, and C3H → C57L would be strongly antigenic. LAF_1 → C57L, Strong A → C3H, C57L → Strong A, and Strong A → C57L would represent good responses. As a cytotoxic

titer was never demonstrated for LAF_1 → Strong A, C3H → LAF_1, and C3H → Strong A, these would be termed poorly antigenic combinations.

The antigens (that is, antigens present in donors and absent in recipients) involved in production of cytotoxic isoantibodies in each of the strain combinations are illustrated in Table VII.

Discussion

From this table, the fact that C3H → Strong A and C3H → LAF_1 did not produce a demonstrable titer appears reasonable. Cann and Herzenberg (22) found that isoantiserum containing antibodies against only two of the component antigens of the H-2 antigenic complexes was cytotoxic. However, there was only one H-2 difference (D^kD^k) in the above strain combinations and this difference did not appear to be associated with strong antigenicity. C3H → Strong A was a notoriously weak combination, very seldom giving demonstrable antibody titers (4,45, 49). Similarly, the poor antigenicity of LAF_1 → Strong A may have been due to insufficient antigenic disparity between donor and recipient when the donor was heterozygous. The same differences, when present in the homozygous form, gave a good antibody response. According to Amos (4), the antibody response to the individual H-2 antigens was not uniform. Neither was the strength of the agglutination reaction necessarily the same for the same antigen on two different cells. This effect may have been due partly to differences in the concentration of antigen and also the physical state of the antigen and of the cell surface. Amos (7) has also stated that not all antigens were equally potent, and some

Table VII

H-2 Antigens Present in the Donor Mice and Absent in the Recipient Mice

Recipient	Donor			
	C3H	C57L	Strong A	LAF_1
C3H	-	D^bD^b FF K^bK^b NN VV	DD FF JJ MM NN	DD^b FF J- K^b- M- NN V-
C57L	AA CC D^kD^k HH KK YY	-	AA CC DD HH JJ KK MM YY	A- C- D- H- J- K- M- Y-
Strong A	D^kD^k	D^bD^b K^bK^b VV	-	D^b- K^b- V-
LAF_1	D^kD^k	-	-	-

antibodies were apparently formed preferentially. Generally, C was not a good antigen. H, J, N and Y were variable, being strong in certain strain combinations and weak in others. A, D, D^b, E, F, K, M and V appeared highly reactive and readily induced antibody production (6).

B. Tissue Reactions

Both donor and recipient mice used in antibody production were 3 to 5 months old at the time of their first injection. After the third to fourth immunizing injection, all experimental mice developed swollen abdomens (Figure 8). These mice became progressively bloated, to the extent that some had difficulty in moving about. A few mice from each experimental group were sacrificed on day 112 for morphological studies. From 5 to 15 ml. of fluid was found in the peritoneal cavity. This fluid varied in composition from copious volumes of a clear, yellowish, slightly oily fluid, to small volumes of a thick, cloudy, yellow, oily fluid. Lieberman (65) has also noted the "swollen" abdomens of mice with ascites fluid formed after 4 to 5 injections of bacteria and adjuvant.

The ascitic fluids were smeared in 20% PVP[a] and stained with Wright's stain. Examination of the smears revealed varying numbers of cells, most of which were polymorphonuclear neutrophils, histiocytes and lymphocytes.

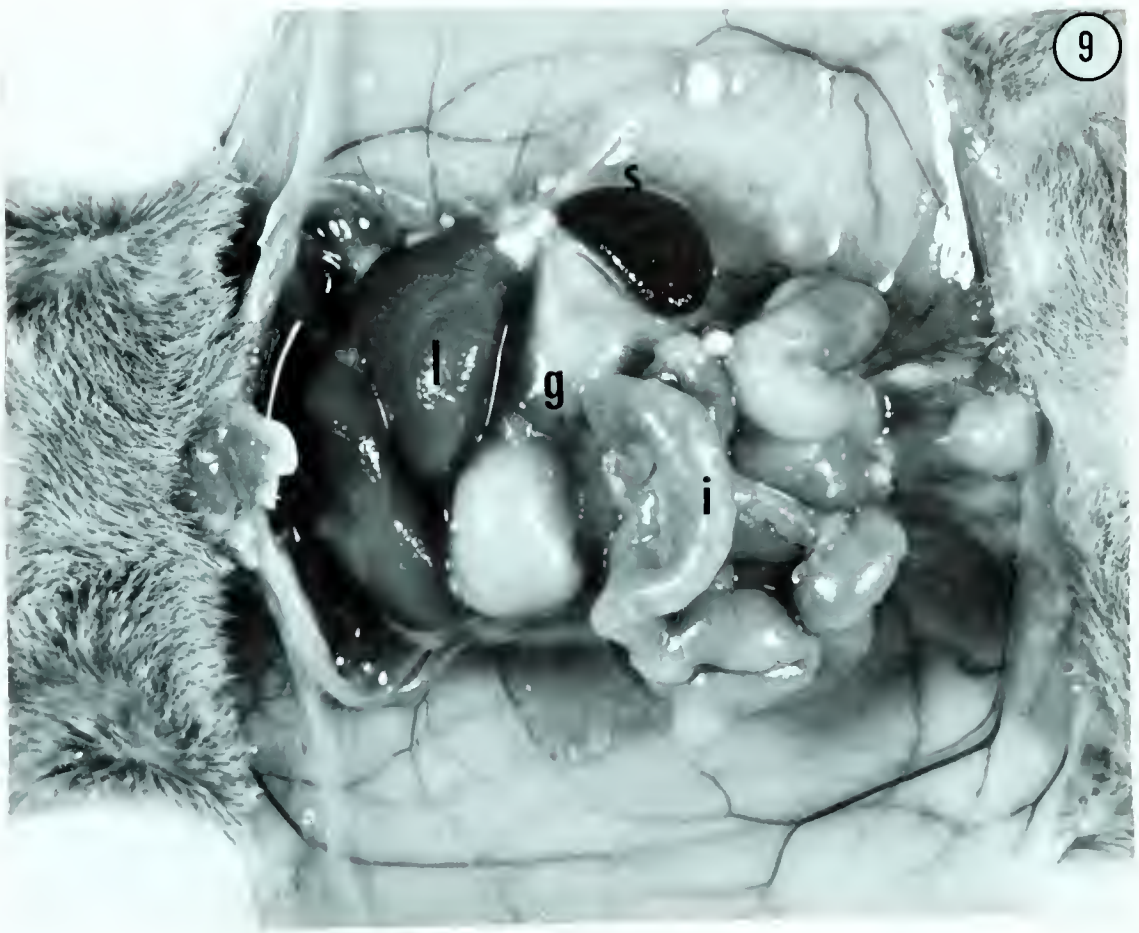
The organs of the abdomen were tightly bound with a white fibrous material (Figure 9). The intestine, stomach, spleen and occasionally the liver and kidneys were covered with this material. The spleen was always grossly enlarged. In the thoracic cavity, the heart, thymus and lungs appeared normal.

[a] Polyvinylpyrrolidone K-30, Chemical Developments of Canada, Toronto.

Figure 8. C3H female mouse immunized with C57L spleen cells and Freund adjuvant, illustrating the swollen abdomen.

Figure 9. Granulomatous reaction (g) in the peritoneal cavity of a C3H mouse immunized with C57L spleen cells and Freund adjuvant. Liver, spleen and intestine indicated by the letters l, s, and i.

8



9

Inguinal and axillary lymph nodes were usually enlarged.

On histological examination,[a] the heart, lungs, thymus, kidneys and intestine appeared normal. The liver displayed hyperplasia of the Kupffer cells, which were increased in plumpness as well as numbers (Figure 10). Many of the Kupffer cells contained vacuoles, which may have been digestive but which were probably vacuoles left by phagocytized adjuvant. A large number of collections of deeply staining cells were present, these foci being mainly paravascular but some being found interspersed amongst the liver cells. The foci were predominately composed of deeply basophilic mononuclear cells with dark staining nuclei, most of which appeared rather primitive. Miller (79) had noticed periportal infiltration of mononuclear cells after intravenous injections of homologous cells. The infiltrates were composed of small cells with deep staining nuclei and cytoplasm, and larger cells with a reticular nuclei and light staining cytoplasm and a few plasma cells. One liver (IVA) also had a great increase in the number of mitoses in parenchymal cells (1 to 2 per high power field).

The spleens always showed hyperplasia of the red pulp, which contained a large number of small dark cells. Loud et al. (66) have also noticed an increase in these cells in the spleen of mice treated with a variety of antigens. These small dark cells were erythroblastic (Figure 11). Megakaryocytes were increased in number. White pulp follicles were also increased in number. The follicles contained germinal centers and were

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[a]The tissues were fixed in formalin and paraffin sections were prepared according to standard laboratory technique. Hematoxylin and eosin staining was used.

Figure 10. Section of liver illustrating a paravascular (p) and a non-paravascular (n) cellular focus. V indicates a blood vessel and K enlarged Kupffer cells. H and E enlarged from x250 mag.

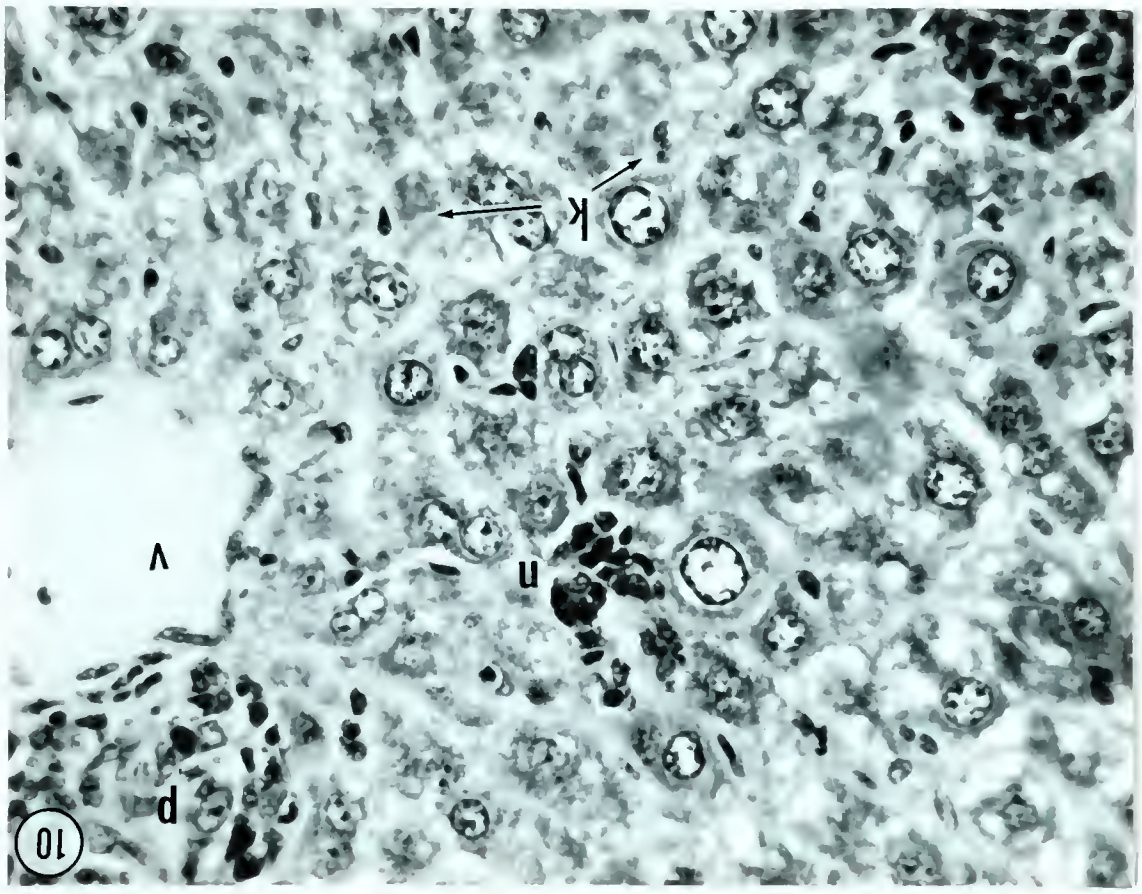
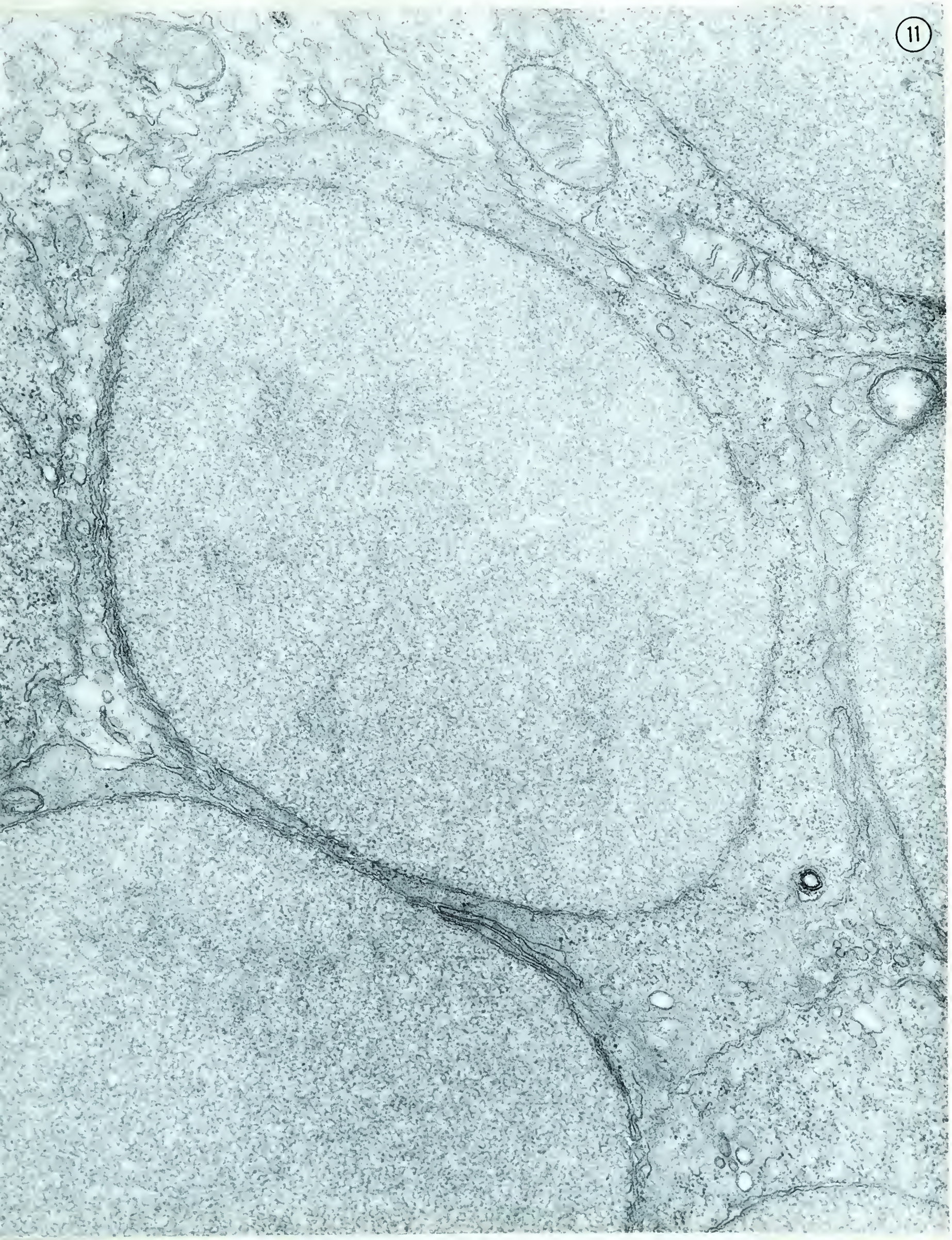

K
V
u
d
10

Figure 11. Electron photomicrograph of red pulp of mouse spleen showing an erythroblast centrally and portions of four other erythroblasts. x45,000 mag.


11

surrounded by a paler peri-follicular area. A large number of cells with dark eccentric nuclei and abundant reddish-blue cytoplasm were present in the spleens (Figure 12). These plasma cells were particularly abundant at the margin of the peri-follicular region, but were also found within the white pulp and in clumps in the red pulp.

The granulomatous reaction surrounding and often extending into the organs of the abdomen, was composed of a great many types of cells. Lymphocytes, polymorphonuclear leukocytes, histiocytes, multinucleated giant cells with fat vacuoles, clumps of plasma cells of all ages, and fibroblasts were seen (Figure 13). The tissue was organizing to fibrous tissue surrounding the holes left by the adjuvant oil.

Lymph nodes characteristically showed hyperplasia of the pulp which contained a few megakaryocytes and a large number of plasma cells of all ages. Lymph follicles were present and they contained an increase in the proportion of primitive cells.

The liver, spleen, lymph nodes, and granulomata were examined for the presence of ribonucleic acid with a methyl green pyronin stain (MGP)[a] on paraffin sections of the tissue. The mesenchymal cells of the liver had a light pink cytoplasm and bluish nuclei. Some of the cells in the foci described above were strongly pyroninophilic (Figure 14). Pyronin positive cells were also present in large numbers in the spleen. The white pulp follicles contained a few positive cells, and

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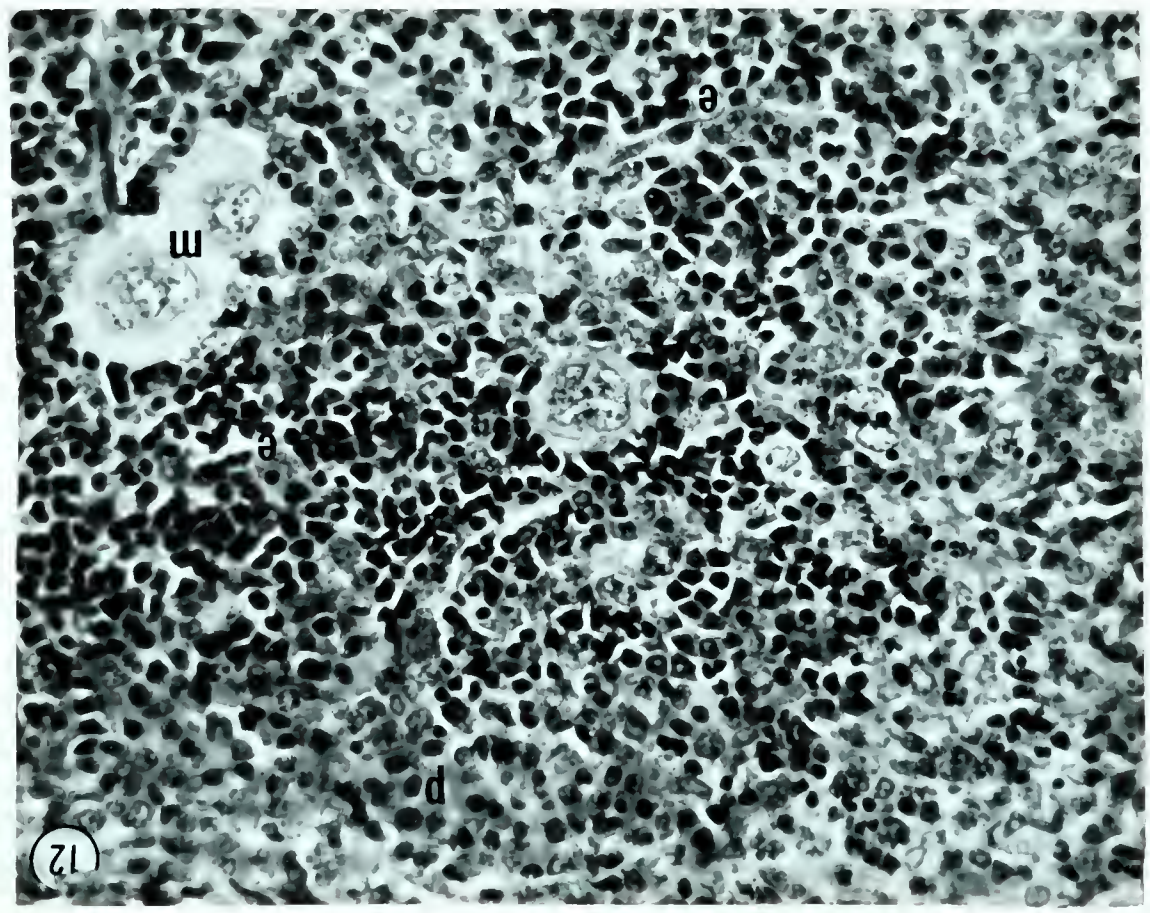
[a]Gurr's, Esbe Laboratory Supplies, Toronto, Canada.

Figure 12. Section of the red pulp of a spleen from an immunized mouse, illustrating an increase in megakaryocytes (m), clumps of plasma cells (p) and groups of erythroblasts (e). H and E enlarged from x250 mag.

Figure 13. Granulomatous reaction about oily vacuoles in the peritoneal cavity. H and E enlarged from x250 mag.


12
p
e
m
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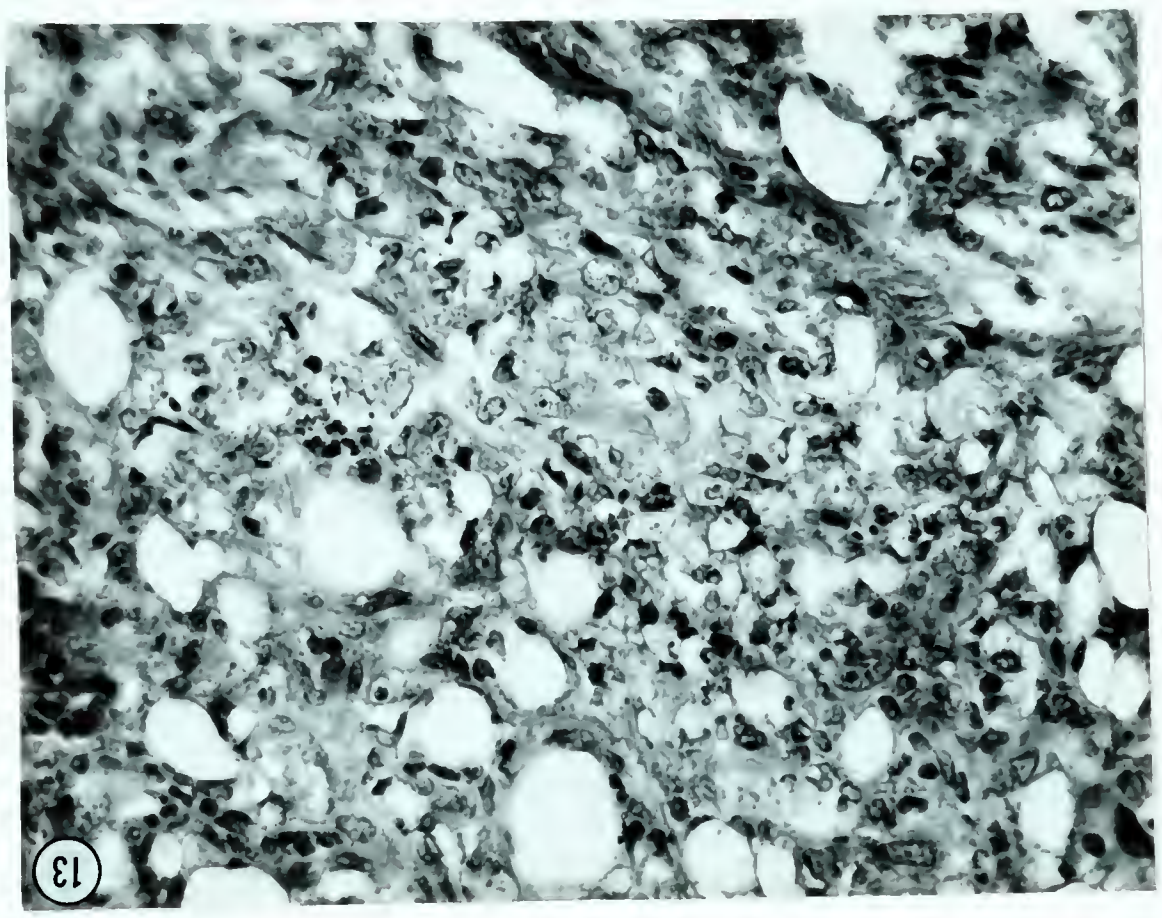

13

the red pulp contained a large number of these cells (Figure 15). A few pyroninophilic cells were found in the white pulp follicles of the lymph nodes. Large numbers of these cells were found in the medullary cords of the lymph nodes (Figure 16). The granulomatous reaction contained clumps of pyroninophilic cells (Figure 17).

Discussion

The reaction in the organs of the immunized mice was primarily confined to the lymphoid tissue, especially the lymph nodes and spleen. The immune response has been reported to begin in the germinal centers of the white pulp of the spleen and cortex of the lymph nodes (24). The cellular changes in the splenic red pulp and lymph node medulla were a result of migration and proliferation of the derivatives of the germinal center cells (24,25). As the immunized mice described in this section were sacrificed on day 112, after six immunizing injections, the preliminary germinal center reaction would not have been observed.

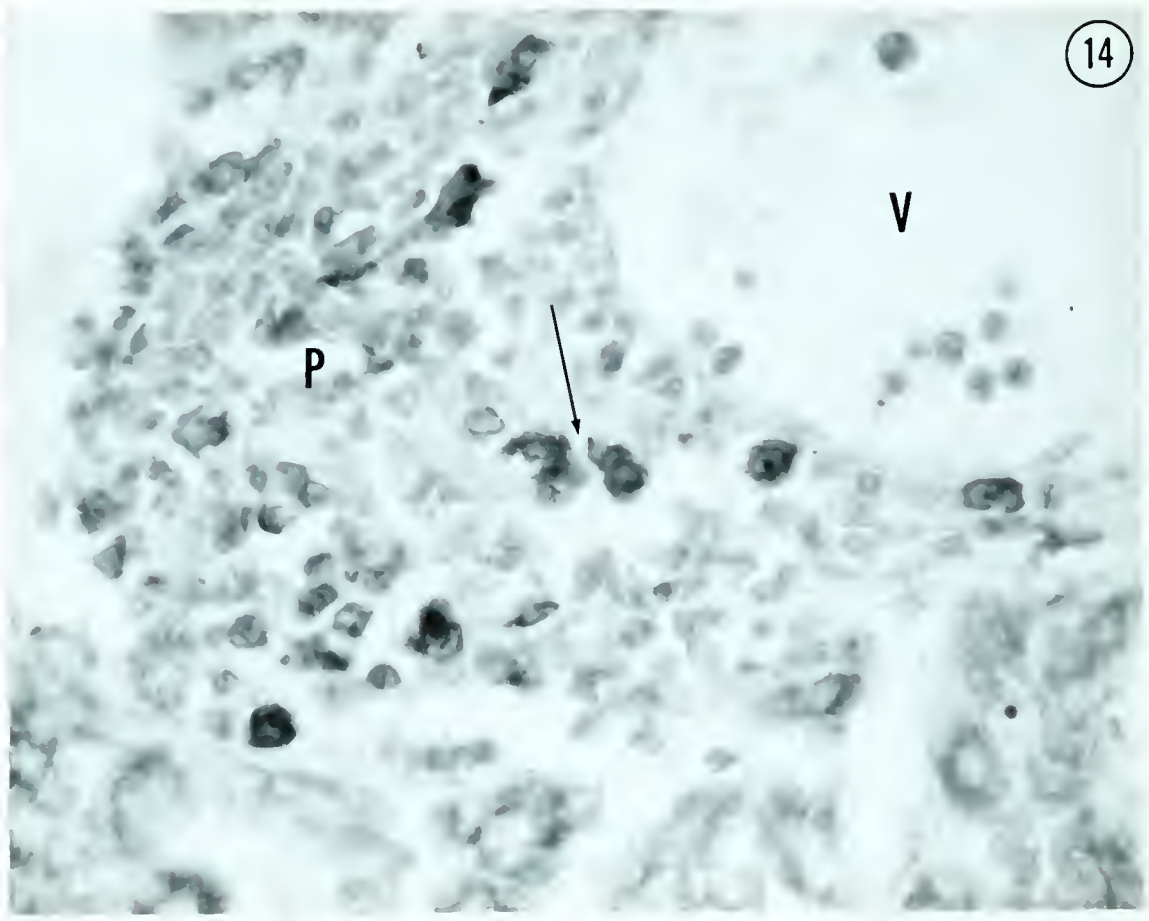
The foci observed in the liver have been reported to be leukocyte infiltrations (27,79). These would not appear to be donor cell infiltrations in these mice, for the greater majority of the mice had high cytotoxic isoantibody titers and it would be unlikely that the donor cells would survive.

C. Examination of Recipient Spleens for Donor Cells

If the hyperplasia of the spleen seen in immunized animals was a result of the deposition of injected cells in the recipient spleen, these cells should be detected in the spleen. To test this hypothesis, two groups were set up. The eight

Figure 14. Pyroninophils (→) in a paravascular focus (p) at the margin of a branch of the portal vein (v) in the liver. Methyl green-pyronin stain, enlarged from x450 mag.

Figure 15. Pyroninophils (→) in a section of red pulp of the spleen. Methyl green-pyronin stain, enlarged from x450 mag.


14
V
P

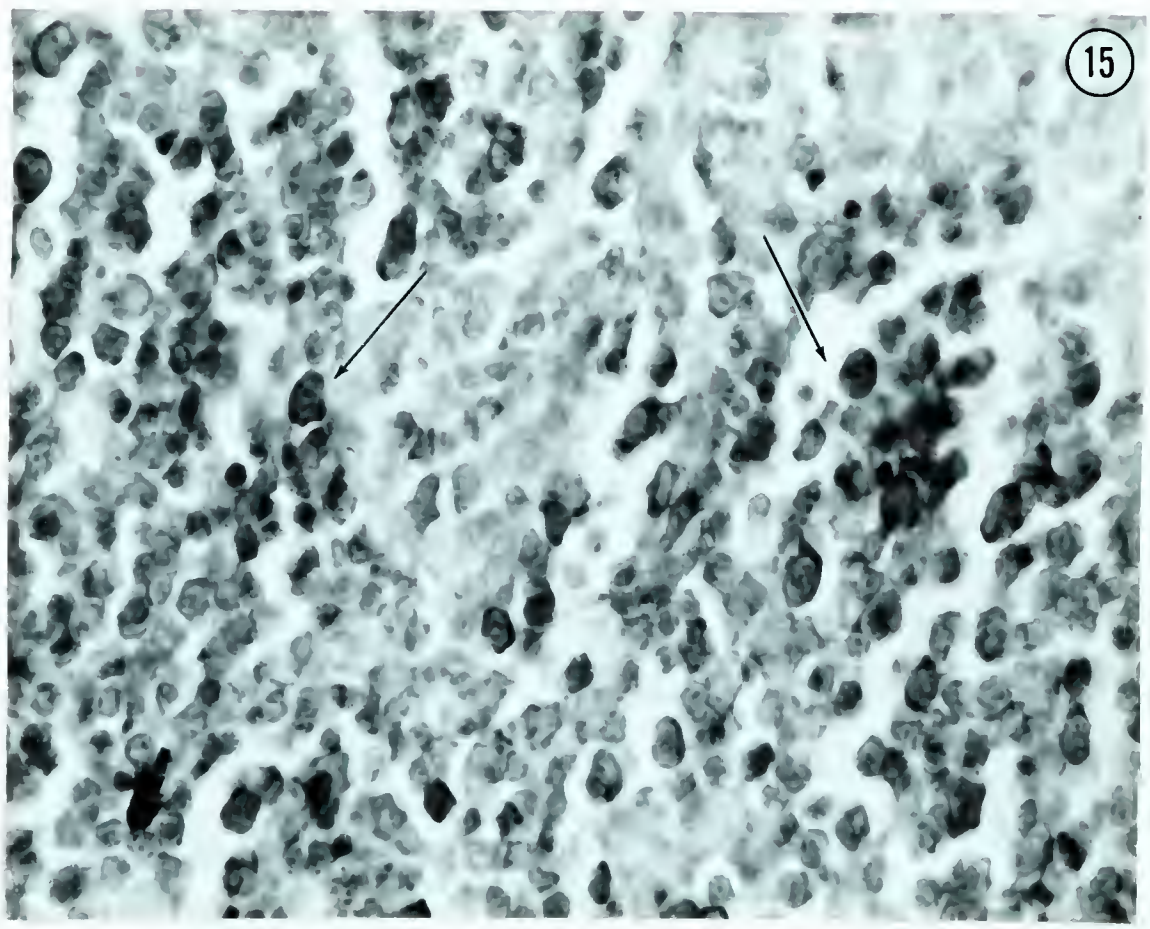

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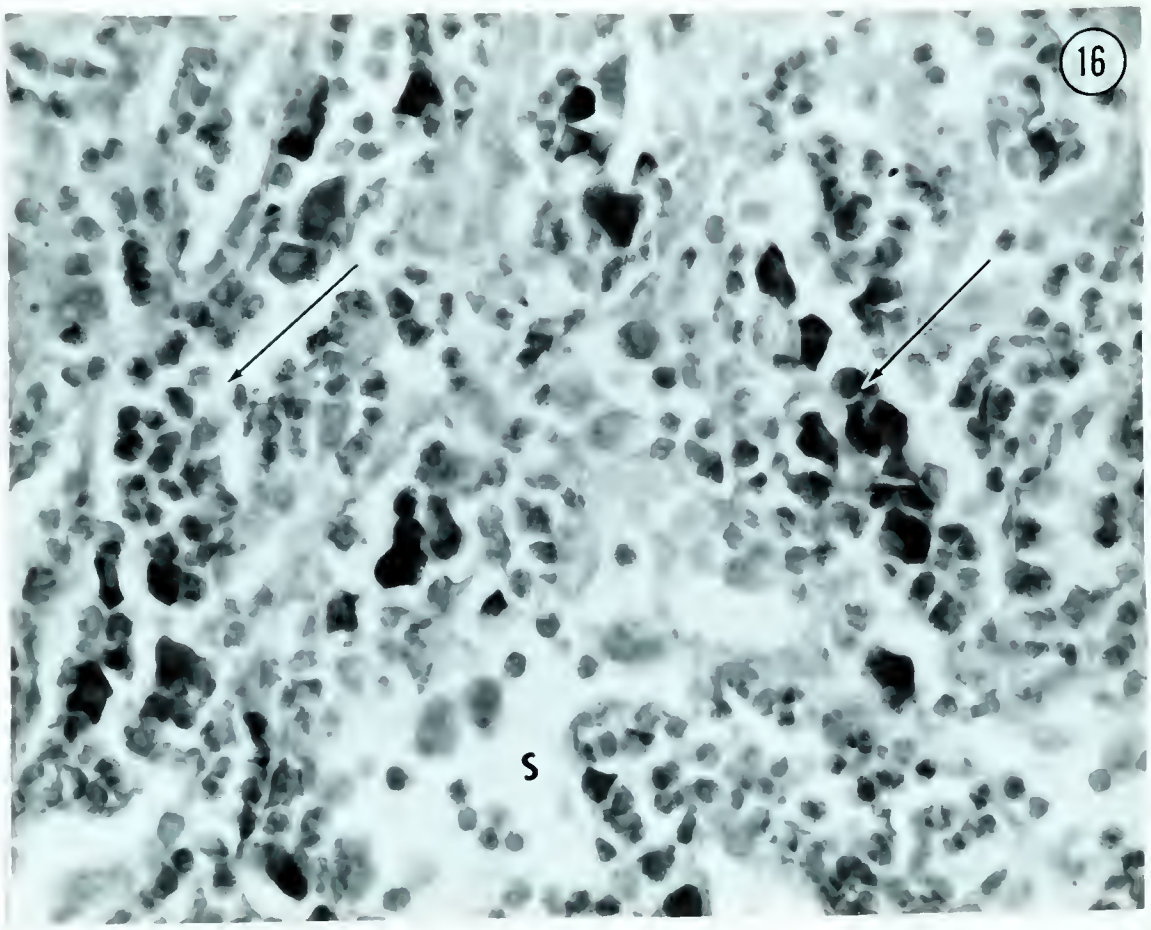
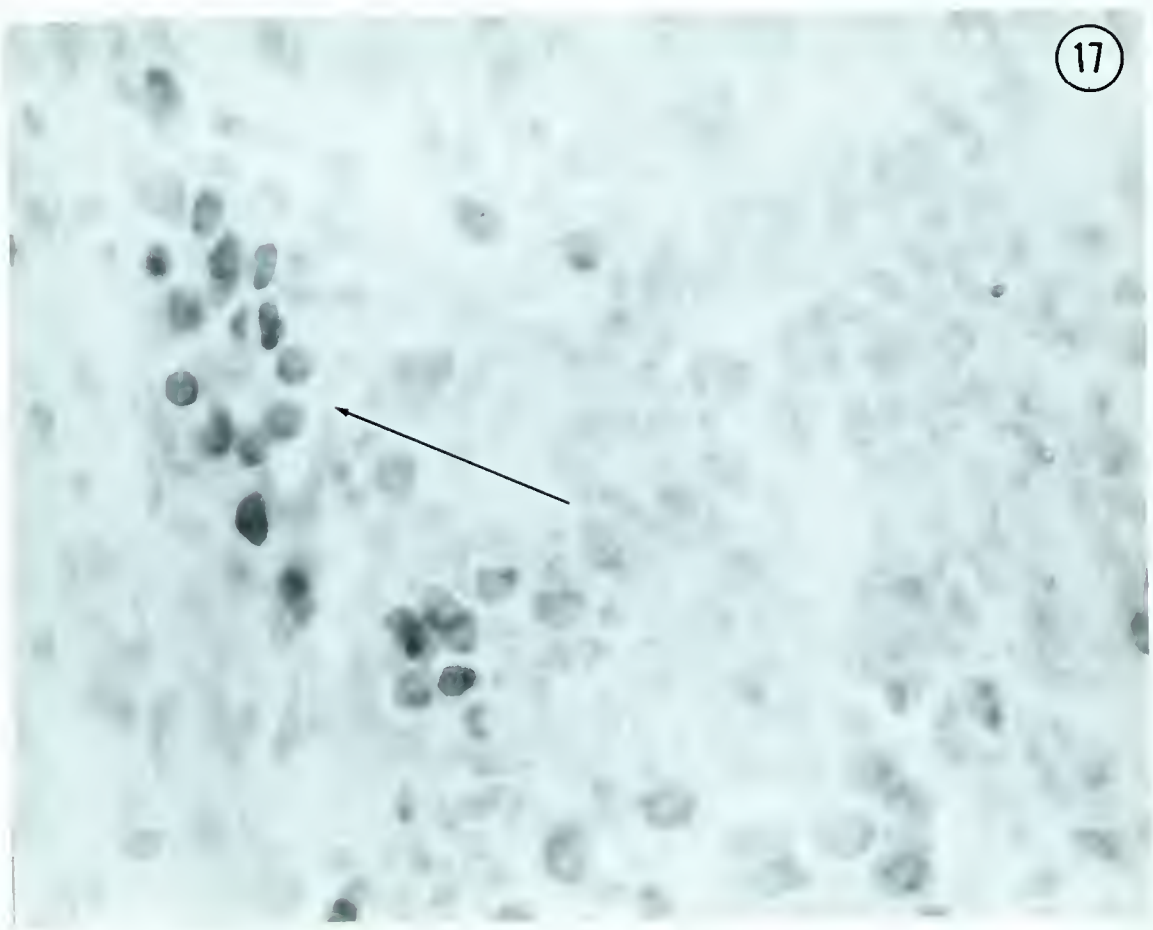
Figure 16. Pyroninophils (→) in the medullary cords of an axillary lymph node. S indicates sinusoids. Methyl green-pyronin stain, enlarged from x450 mag.

Figure 17. Pyroninophils (→) present in granulomatous reaction. Methyl green-pyronin stain, enlarged from x450 mag.


16
S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female C57L mice (three months old) of Group One were each injected intraperitoneally with a half spleen dose of normal female C3H cells (2×10^8 cells). The eight C57L female mice of Group Two received a half spleen dose (2×10^8 cells), from a C3H female mouse who had received an intraperitoneal injection of 5.5×10^7 C57L female spleen cells the week before. One mouse from each group was sacrificed on days 1, 3, 5, 8, 10, 14 and 26; a normal C3H and a normal C57L mouse were also sacrificed for normal cell controls. Spleen cell suspensions of each were prepared as described previously.

Aliquots of the test spleen from Group One and Group Two were incubated with high titered C3H anti C57L and a C57L anti C3H serum and 1:8 guinea pig complement. The proportion of stained cells was determined as described before (page 21). Twelve controls were also titrated, so that each day's test included all possible combinations of normal C3H serum, normal C57L serum, C3H anti C57L serum and C57L anti C3H serum with normal C3H, normal C57L, Group One, and Group Two spleen cells. In addition, C57L anti C3H and C3H anti C57L serum were tested against mixtures of equal numbers of normal C3H and normal C57L spleen cells prepared in vitro.

The percent relative mortalities of the positive control ranged from 60 to 98, and were usually between 70 and 90. In the negative controls, the percent relative mortalities did not exceed 4. The results (Table VIII) of tests on the spleens of Group One and Group Two were expressed as fractions of the expected percent relative mortality as determined from the corresponding positive control.

Table VIII

Proportion of Spleen Cells Susceptible to Specific Cytotoxic Antiserum

Days after Spleen Cell Transfer	Group One		Group Two	
	C3H anti C57L serum	C57L anti C3H serum	C3H anti C57L serum	C57L anti C3H serum
1	0.94	0.01	1.00	0.01
3	1.02	0	0.87	0
5	1.02	0	1.08	0.02
8	1.03	0.12	1.01	0
10	0.82	0.01	0.91	0
14	0.96	0.03	1.01	0.05
26	0.85	0.03	0.95	0.02

Discussion

These results indicate that if the donor C3H spleen cells migrated to the spleens of the C57L animals, they did so in such small numbers that they could not be demonstrated by the cytotoxic isoantibody method. It would appear that the increase in splenic weight of immunized mice was not due to the proliferation of injected C3H cells in the host C57L spleens but was primarily due to proliferation of host spleen cells. These results were in contrast to those obtained by Boyse (17) and Fox (33) when parental stain cells were injected into F_1 hybrids. They found the donor cells showed an initial proliferation and then settled to diminished small numbers which persisted from 2 to 8 weeks.

D. Demonstration of Isoantibodies by Three Methods

Tables IX and X illustrate the log titers obtained from certain antisera by three methods: modified spleen cell cytotoxic isoantibody titration of Gorer and O'Gorman (46), the human serum-dextran red cell agglutination technique of Gorer and Mikulska (47), and a saline red cell agglutination titration. These methods were described in detail in Section III.B.

Discussion

IIB and IVA may be regarded as comparable to the extent that the same single homozygous allele was foreign and consequently antigenic. A anti C3H was a notoriously weak antiserum and was usually non-reactive (4,45,47,49). Neither serum contained isoantibody demonstrable with saline red cell agglutination (S) or cytotoxic isoantibody methods (C). Both contained red cell agglutinins when tested by the human serum-dextran

Table IX

Comparison of the Isoantibody Titers Obtained with Various Inbred Mouse Strain Combinations

Antiserum	Foreign Antigens	*	Log_2 Titer								
			Days					Months			
			25	82	92	102	112	7	7-1/2	9	9-1/2
IVB	DD FF JJ	C	0	9.4	9.5	11.3	-**	9.0	-	0	0
C3H anti A	MM VV	HSD	7.0	8.0	10.0	-	-	8.0	-	8.0	10.0
		S	0	-	-	-	-	-	-	-	0
IIA	DD^b FF J-	C	4.9	9.1	8.5	9.9	9.6	-	-	-	-
C3H anti LAF_1	K^b- M- NN V-	HSD	-	14.0	14.0	14.0	15.0	-	-	-	-
		S	-	-	0	0	-	-	-	-	-
IA	D^bD^b FF	C	6.8	10.6	10.3	10.7	10.7	-	10.0	-	-
C3H anti C57L	K^bK^b MM VV	HSD	6.0	10.0	-	11.0	11.0	-	0	-	-
		S	0	0	-	0	-	-	-	-	-
CLB		C	0	5.1	6.3	8.5	8.6	-	-	-	-
C3H anti C57L		HSD	0	0	4.0	8.0	9.0	-	-	-	-
(lyophilized		S	0	0	0	0	0	-	-	-	-
IB	AA CC D^kD^k	C	3.1	6.3	5.7	6.3	5.8	-	-	5.8	-
C57L anti C3H	HH KK YY	HSD	-	9.0	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-	-	-

*C - Cytotoxic isoantibody titration.
HSD - Human serum-dextran agglutination.
S - Saline agglutination.
**- Not tested.

Table X

Comparison of the Isoantibody Titers Obtained with Various Inbred Mouse Strain Combinations

Antiserum	Foreign Antigens	*	Log_2 Titer								
			Days					Months			
			25	82	92	102	112	7	7-1/2	9	9-1/2
VB	AA CC DD	C	0	8.4	10.7	9.8	12.0	9.2	-	9.3	-
C57L anti A	HH JJ KK	HSD	-	13.0	15.0	-	17.0	13.0	-	13.0	-
	MM YY	S	-	7.0	9.0	9.0	-	-	-	8.0	-
IIIB	A- C- D-	C	0	6.2	8.8	8.7	8.3	-	-	-	-
C57L anti LAF_1	H- J- K-	HSD	-	-	-	13.0	17.0	-	-	-	-
	M- Y-	S	-	-	-	0	0	-	-	-	-
IIIA		C	0	0	0	0	0	-	-	-	-
A anti LAF_1	D^b- K^b- V-	HSD	-	0	-	4.0	9.0	-	-	-	-
		S	-	0	-	0	0	-	-	-	-
VA		C	0	5.6	-	8.8	10.0	9.9	-	9.9	-
A anti C57L	D^bD^b K^bK^b	HSD	0	6.0	6.0	9.0	13.0	13.0	-	8.0	-
	VV	S	0	0	0	0	0	0	-	-	-
IIB		C	0	0	0	0	0	-	-	-	-
LAF_1 anti C3H	D^kD^k	HSD	-	0	6.0	9.0	6.0	-	-	-	-
		S	-	0	0	0	0	-	-	-	-
IVA		C	0	0	0	0	0	-	0	-	0
A anti C3H	D^kD^k	HSD	8.0	0	8.0	9.0	-	-	0	-	0
		S	0	-	0	0	-	-	0	-	0

* C - Cytotoxic isoantibody titration.
HSD - Human serum-dextran agglutination.
S - Saline agglutination.
\- - Not Tested.

method (HSD). This may indicate a different isoantibody not detectable by the C method or may simply reflect the increased sensitivity of HSD as compared to C (5,49,58), for according to Gorer (42), C3H red blood cells were not agglutinated unless a very high titered antiserum was used.

It would appear reasonable that homozygous alleles would be more effective in the production of isoantibody than heterozygous alleles. The effect was more pronounced in the HSD series, where homozygocity (VA and VB) resulted in the demonstration of isoantibody earlier and in higher titer than the respective heterozygous counterparts (IIIA and IIB). The same effect was shown for IIIA and VA in the C titration. However, in VB and IIIB, although the isoantibody titers were higher when the alleles were homozygous, isoantibody could be demonstrated for both homozygous and heterozygous sera on the same day. This may be due to relatively greater disparity at the H-2 locus. VB (Figure 18) was the only serum with a demonstrable S, which may be due in part to the observation that strain A red blood cells were extremely sensitive to agglutination. It would appear then, that the C test measured both saline agglutinating and saline non-agglutinating antibody, and that the disparity between HSD and C was due to the increased sensitivity of the HSD method. However, this generalization does not hold true throughout, for the C and HSD titers of VA were approximately the same.

It has been reported that repeated immunization may result in lower titers of agglutinating antibody and increased

titers of C (91). This does not appear to be the case in any of the sera tested above. In one instance (VA), the C and HSD titers were the same, rising and falling together. In another (IA), isoantibody demonstrable by the C and HSD method, appeared at the same time and were of the same magnitude, but the HSD titer fell off first. In IVB the HSD isoantibody appeared first and was still present when C was no longer demonstrable, but at times when both were demonstrable they were of the same magnitude. VB, IIB and IIA all had higher HSD than C titers, and persistence appeared to be about the same. IIIA had no demonstrable C and a late appearing, comparatively strong HSD. CLB sera contained comparable HSD and C titers; however, the HSD appeared later. IIB and IVA had no isoantibody which could be demonstrated by C, and a rather unusual HSD. The HSD titer appeared early, fell and then rose again.

Summary

The above results indicated that the demonstrable hemagglutinating isoantibodies (HSD) frequently paralleled the cytotoxic isoantibodies (5,58). In most cases, HSD demonstrated higher titers than C (5,49,58). It has been suggested that both methods probably measure the same isoantibody (20,58,80,107). However, antibodies demonstrable by C were not necessarily demonstrable by HSD, and vice versa (113).

E. Failure of a Nonreactive Serum to Inhibit Cytotoxic Activity of a Potent Antiserum

Some of the strain combinations did not result in the production of a detectable cytotoxic isoantibody with the modified Gorer and O'Gorman (46) technique. One of these sera (IIIA)

was tested with a comparable strong reacting antiserum (IIA) to investigate the possibility that the non-reacting serum may be inhibitory.

C3H anti LAF_1 serum (IIA) with a cytotoxic $\log_2$ titer of 10.0 was mixed with an equal volume of Strong A anti LAF_1 serum (IIIA). The combined sera were titrated against LAF_1 spleen cells (2×10^7/ml.) as described on page 21. If IIIA serum was not inhibitory to IIA serum, the cytotoxic $\log_2$ titer should be one $\log_2$ unit lower than that of IIA serum titrated by itself. Examination of the test for proportions of stained cells resulted in a $\log_2$ titer of 8.8. The non-reactive serum was therefore not inhibitory to the cytotoxic activity of strongly cytotoxic serum.

F. Comparison of Lyophilized and Fresh Spleen Cells as Immunizing Agents

A comparison of living fresh tissue and lyophilized tissue as an immunizing agent was made, for living tissue has been reported to be superior to non-living tissue as an immunizing agent (58,91,94).

Two experimental groups were set up; one was immunized with fresh spleen cells and adjuvant according to the schedule on page 14; and the other with lyophilized spleen cells and adjuvant as described on page 15.

The relative effectiveness of the two forms of immunization on the production of isoantibody demonstrable by the cytotoxic isoantibody method (page 21) is illustrated in Figure 19. Lyophilized spleen cells and adjuvant did not result in

Figure 18. Isoantibodies in C57L anti A serum demonstrable with human serum-dextran (□) and saline (▣) hemagglutination techniques and with cytotoxic isoantibody titration (● ——). Arrows indicate immunizing injections.

Figure 19. Comparison of the effectiveness of lyophilized (0) and fresh (●) tissue in stimulating cytotoxic isoantibodies. Arrows indicate immunizing injections.

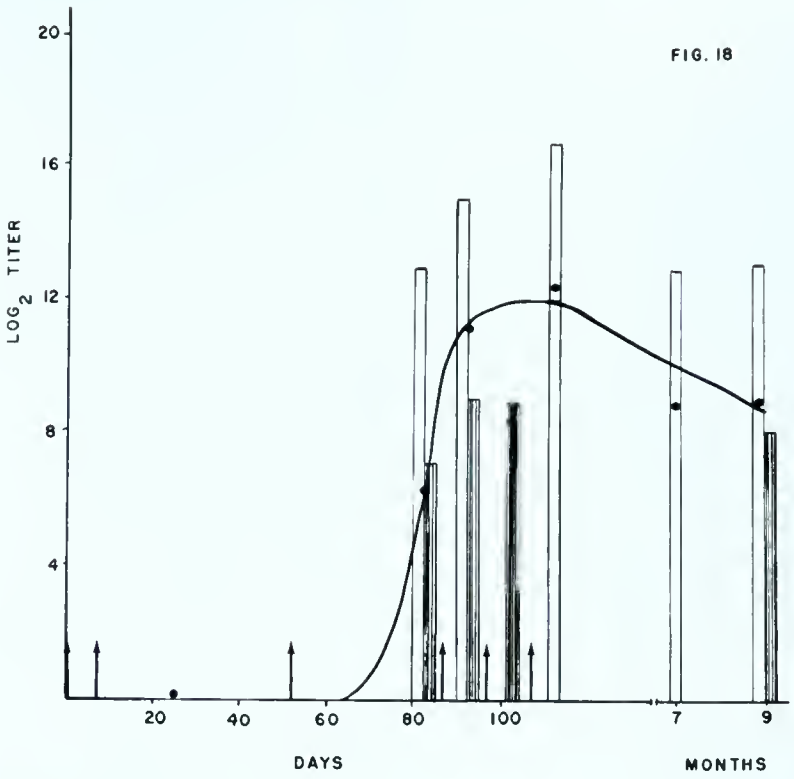
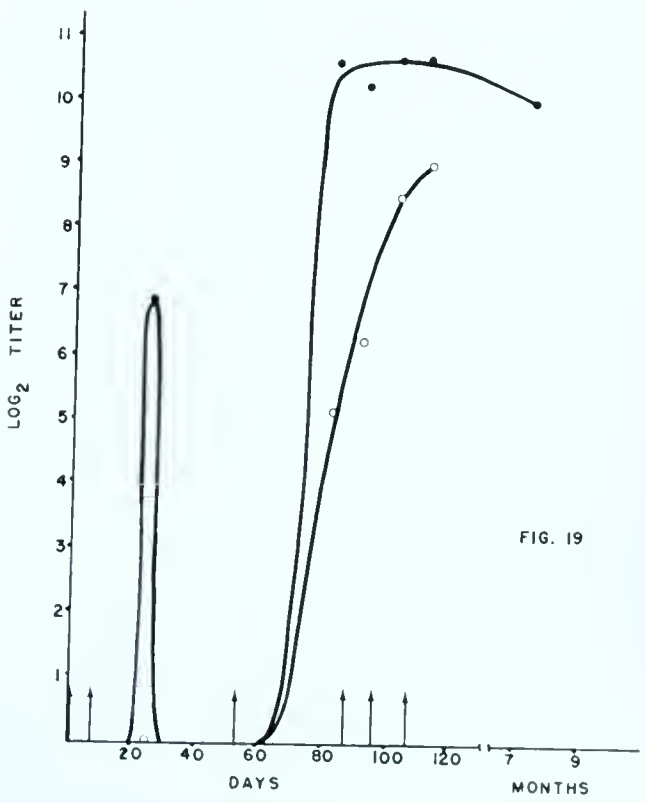

20
16
12
8
4
LOG2 TITER
20
40
60
80
100
7
9
DAYS
MONTHS

FIG. 18


11
10
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8
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6
5
4
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2
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LOG2 TITER
20
40
60
80
100
120
7
9
DAYS
MONTHS

FIG. 19

the early 25 day primary peak and although the titer climbed steadily with subsequent immunizing injections, it never reached the levels produced with fresh spleen cells.

Red cell agglutinations as demonstrated by the human serum-dextran method (HSD) were also lower and appeared later (Table IX) in the lyophilized test series. Pizarro (94) also found the HSD titers of mice injected with fresh tissue to be constantly higher than the HSD titers of mice injected with lyophilized tissue. However, she found that the persistence and decline of the antibody was the same for the two groups.

G. Relative Effectiveness of Antiserum and Immune Lyophilized Spleen as Cytotoxic Agents

The spleen of a C3H mouse immunized with C57L spleen cells and adjuvant was removed aseptically on day 112. A spleen suspension was prepared as described previously; 2.0 ml. aliquots of which were lyophilized as described on page 15. Before reconstitution with 2.0 ml. of sterile distilled water, the lyophilized aliquot was ground with a sterile mortar and pestle. The suspension was then centrifuged at 1725 x g for 10 minutes and the supernatant removed. The supernatant was treated as an antiserum and a cytotoxic titration was set up (page 21) with C57L spleen cells. The C57L spleen cells were also titrated with a C3H anti C57L serum ($\log_2$ 9.9). The immune spleen cell supernatant did not give a demonstrable level of cytotoxicity, while a $\log_2$ titer of 9.8 was obtained with C3H anti C57L serum.

These results were in contrast to those of Wilson and Crosby (120), who found that immune lymph node and spleen cells

were cytotoxic in all subcellular fractions. They found that the portion containing nuclear debris, cell walls mitochondria and the supernatant fraction had the highest activity. Complement did not appear to be necessary, and heat destroyed the activity of all the subcellular fractions.

VI. TISSUE AND AGE SENSITIVITY

Sensitivity to cytotoxic isoantibody is a direct function of the concentration of H-2 antigens on the cell's surface (80,90), and is well-correlated with the absorbing capacity of the cells (80,84). It is conceivable then, that all cell types would be sensitive to the action of cytotoxic antiserum, provided the antigen-antibody-complement complex could be supplied in the proper quantities (80,107). This is illustrated by the action of two antisera (one of which is very weak) produced against tissues of the same strain. A mixture of the two antisera will give a higher titer than the more potent antiserum acting by itself. Therefore, the activity of an antiserum is not solely dependent on the total amount of isoantibody present, but also on the availability of isoantibody combinations of certain specifications (80,84,124).

However, there is a variation in the sensitivity of different cells to the cytotoxic action of antisera in vitro (67,80,81,90,91,107,113,124). Generally, normal spleen (43, 91), lymph node (43,80,91) and bone marrow (43) are highly sensitive and thymus cells (90,91,113,124) are resistant. Thymocytes have also been reported to fix far less complement than lymph node, spleen and bone marrow (90). Newborn

cells of all types are resistant to the cytotoxic action of isoantisera (81,93). The increase in sensitivity of the newborn cells with age is well-correlated with an increase in the concentration of surface receptors on these cells (83,93).

C3H tissues have been reported to be insensitive to the action of specific isoantiserum until the age of 2-1/2 days. Adult levels were reached at 6 days (81,93). In an attempt to confirm these findings, C3H male and female mice at various ages, and a C57L anti C3H serum were used. Two to four mice were sacrificed at various ages. The thymuses, spleens and pooled axillary and inguinal lymph nodes were each suspended by teasing with sterile needles. The cell suspensions were adjusted to approximately 2×10^6 cells/0.1 ml. and held at 4°C. for one hour. Each cell suspension was tested for susceptibility to C57L anti C3H serum according to the standard technique (page 21). The same antiserum (C57L anti C3H day 82, $\log_2$ titer 7.0) was used throughout. Table XI summarizes the results of this experiment.

Discussion

The spleen showed the earliest sensitivity to cytotoxic isoantibody; however, adult levels were not obtained until after seven weeks. The lymph nodes also showed a sharp rise in sensitivity, reaching adult levels some time after seven weeks. Thymus cells were almost completely resistant throughout the time tested. These results were in keeping with those obtained by others, in regard to tissue sensitivity. However, in contrast to the findings of Möller

Table XI

Tissue and Age Sensitivity of C3H Reticulo-Endothelial Tissue Cells to the Action of Cytotoxic Isoantibody

Age (weeks)	Log_2 titer (50% end point)		
	Spleen	Thymus	Pooled Lymph Nodes
2	3.0	<1	1.0
3	3.0	<1	3.0
5	4.0	<1	5.0
7	5.8	<1	6.6
9	7.0	<1	7.5

(81) and Pizarro (93), adult levels were not reached until between seven and nine weeks. Möller (81) used a different method to calculate the cytotoxic isoantibody titer, whereby the highest dilution yielding a cytotoxic index of ≥ 0.15 was taken as the titer. The cytotoxic index was the proportion of unstained cells in the control minus the proportion of unstained cells in the test, divided by the proportion of unstained cells in the control. Möller did not indicate the proportion of unstained cells in the control. However, if 96% unstained cells were present in the control, approximately 80% would have to be present in the test to obtain a cytotoxic index of ≥ 0.15. A typical cytotoxic titration curve plotted from the percent relative mortalities (Figure 3) illustrates a leveling off of the percent relative mortality at approximately 35. We have also noted that 30% of cells dead in normal serum dilutions below 1:16 (Figure 1). If Möller's method of calculating the cytotoxic titer were used, the cytotoxic index would be approximately 0.15 for all the antiserum dilutions above the 50% relative mortality end point. Perhaps the apparent sensitivity of neonatal cells to cytotoxic isoantibody is merely a reflection of the non-specific death of cells due to dilution.

Summary

Adult sensitivity of spleen and lymph node cells to specific cytotoxic isoantibody was reached between 7 and 9 weeks.

The cells of the thymus were resistant to the action of cytotoxic isoantibody and complement.

VII. COMPLEMENT

Low levels of complement have been demonstrated in mouse sera (64,73); however, the *in vitro* cytotoxic activity of mouse antiserum has never been demonstrated by the Gorer and O'Gorman method (46), unless heterologous serum was added to the test system as a source of complement (5,10,14, 22,45,80,101,124). Gorer (43) quoted Ritz as saying in 1911 that the "end piece" of mouse complement (C') was missing, and Brown in 1943, that C'2 was absent. More recently, Rice (98) has shown that mouse sera contain considerable amounts of C'1, low concentrations of C'2 and C'3 not demonstrable in dilutions past the anti-complementary range, and no demonstrable C'4. Thus the treatment of target tissue cells with specific antiserum alone was not lethal to the cells (22,52, 60,122), although high concentrations of antiserum did inhibit target cell growth in tissue culture (52,81). High concentrations of guinea pig sera were lethal to cultured normal mouse tissue cells and resulted in an increased number of stained cells in the normal serum control (22).

A. Complement Dependence of the Cytotoxic Isoantibody Titration

The following experiment illustrated the dependence of the modified cytotoxic isoantibody titration of Gorer and O'Gorman (46) on the presence of heterologous active complement. A potent C57L anti LAF_1 serum was titrated against

normal LAF_1 spleen cells according to our standard technique (page 21). The antiserum was not cytotoxic to LAF_1 cells when the added guinea pig complement had been previously inactivated in a water bath at 56°C. for one hour, or when normal LAF_1 serum, or PBS were substituted for active complement. When titrated with active 1:8 guinea pig complement in PBS, a $\log_2$ titer of 8.2 was demonstrable.

The complement requirement for the demonstration of maximum lethality of a cytotoxic antiserum was illustrated in the following experiment utilizing a checkerboard system. The columns contained dilutions of C3H anti C57L serum (1:1 to 1:1024) and a normal undiluted C57L serum control, while the rows contained dilutions of complement (1:1 to 1:1024). C57L spleen cells (2×10^7/ml.) were added to each tube and the titration carried out as described on page 21. The $\log_2$ titer (50% relative mortality end point) was plotted against the complement dilution (Figure 20). Adequate complement was present for maximum lethality at complement dilutions of 1:2 to 1:64. Undiluted complement was inhibitory to cytotoxicity. This inhibition, attributed partly to a naturally occurring anti-mouse-antibody in normal guinea pig serum, increased the cell death in the control (22,113) and consequently decreased the percent relative mortality. Also, high concentrations of complement have been reported to be inhibitory to cytotoxic isoantibody (22). Dilutions above 1:64 did not provide sufficient complement for maximum action of the cytotoxic isoantibody.

Figure 20. Titers obtained with C3H anti C57L serum titrated against C57L cells with varying concentrations of complement.

Figure 21. Anti-sheep red blood cell hemolysin-complement titration using 2% washed sheep red blood cells.

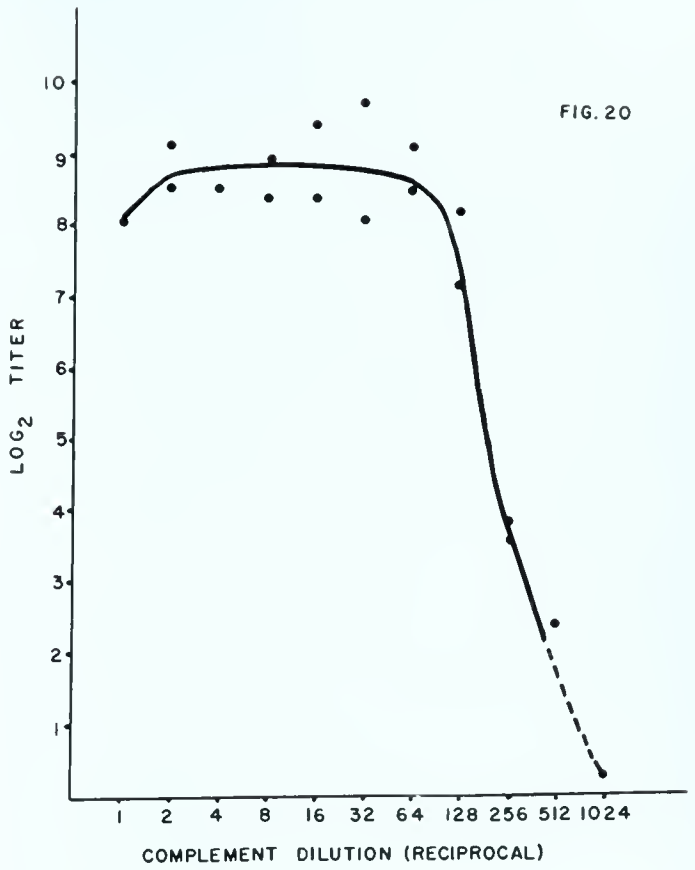


FIG. 20
LOG_2 TITER
10
9
8
7
6
5
4
3
2
1
1 2 4 8 16 32 64 128 256 512 1024
COMPLEMENT DILUTION (RECIPROCAL)

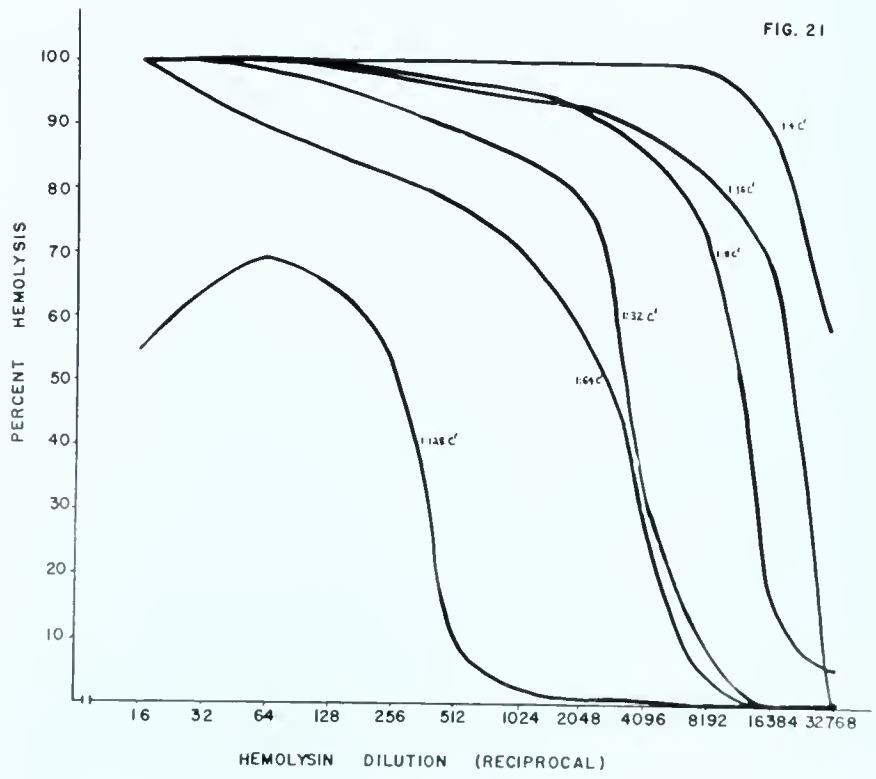


FIG. 21
PERCENT HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
16 32 64 128 256 512 1024 2048 4096 8192 16384 32768
HEMOLYSIN DILUTION (RECIPROCAL)

B. Hemolytic Indicator System

The complement requirement of the hemolysin system was analyzed in the following experiment. A checkerboard system was set up, the columns containing 0.1 ml. aliquots of complement dilutions (1:1 to 1:2048) and the rows 0.1 ml. aliquots of hemolysin dilutions (1:1 to 1:32768). To all tubes were added 0.2 ml. of Kolmer saline and 0.1 ml. of 2% sheep red blood cells, which were then incubated at 37°C. in a water bath for 30 minutes. The amount of hemolysis was determined as previously described (page 27). In hemolysin dilutions of 1:1 to 1:8, complete hemolysis was noted with complement dilutions of 1:1 to 1:4. Complement dilutions of 1:1 to 1:4 showed complete hemolysis for all hemolysin dilutions except 1:32768. Dilutions of complement from 1:256 to 1:2048 were insufficient for complete hemolysis at any hemolysin dilution. The remaining results are illustrated in Figure 21, where hemolysin dilution is plotted against percent hemolysis.

C. Complement Fixation in the Cytotoxic Isoantibody System

The complement utilization of cytotoxic isoantibody was further investigated according to the principle that complement fixation can be more accurately determined if an excess of complement is used and residual complement determined with a hemolytic system (12,88,124). The residual complement left after the cytotoxic isoantibody and target spleen cells had reacted in the presence of complement was measured by the addition of a hemolytic system. A checkerboard

system was used as described above, and the hemolysin system (0.1 ml. of 2% sheep red blood cells and 0.1 ml. of 1:1000 rabbit anti sheep hemolysin) added after the first 30 minutes of incubation. Adequate controls (page 26) were included. The percent hemolysis was calculated and corrected as described on page 27. The results of such a titration are illustrated in Figure 22, where percent hemolysis was plotted against isoantiserum concentration. There was enough complement left over to result in complete hemolysis when the complement concentration was between 1:1 and 1:32. A slight decrease in the amount of hemolysis was noted with low dilutions of isoantiserum. This decrease was probably due to the anticomplementary activity of low dilutions of mouse serum (5,43, 124). The results have been corrected for serum color. Complement dilutions above 1:32 did not result in complete hemolysis. However, hemolysis increased with increasing antiserum dilution, providing strong evidence for the complement utilization of the system. A 1:8 complement dilution was chosen as the optimum concentration of complement for use in the cytotoxic isoantibody titration. This complement concentration was well in excess of that required for demonstrating maximum cytotoxicity and approximately at the 4% limit suggested by Cann and Herzenberg (22) to avoid the natural anti-mouse activity of guinea pig serum. A 1:8 complement was also below that concentration which was inhibitory to cell death (Figure 20).

Figure 22. Complement fixation of A anti C57L serum-C57L spleen cell system at complement dilutions of 1:2 to 1:512, with 1:1000 hemolysin and 2% sheep red blood cells.

Figure 23. Comparison of the complement fixation by 2% sheep red blood cells and 1:1024 anti sheep hemolysin (●) with that produced by 2% sheep red blood cells and 1:1000 anti sheep hemolysin in presence of 1:1024 A anti C57L serum and C57L spleen cells (o).

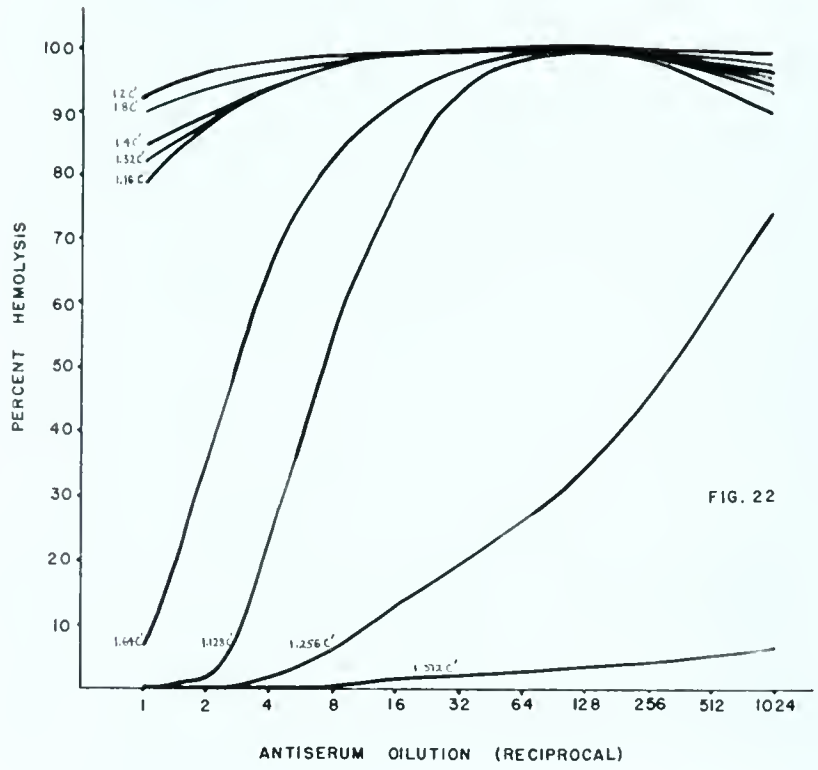

PERCENT HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
1.2 C'
1.8 C'
1.4 C'
1.32 C'
1.16 C'
1.64 C'
1.128 C'
1.256 C'
1.512 C'
1
2
4
8
16
32
64
128
256
512
1024
ANTISERUM DILUTION (RECIPROCAL)

FIG. 22

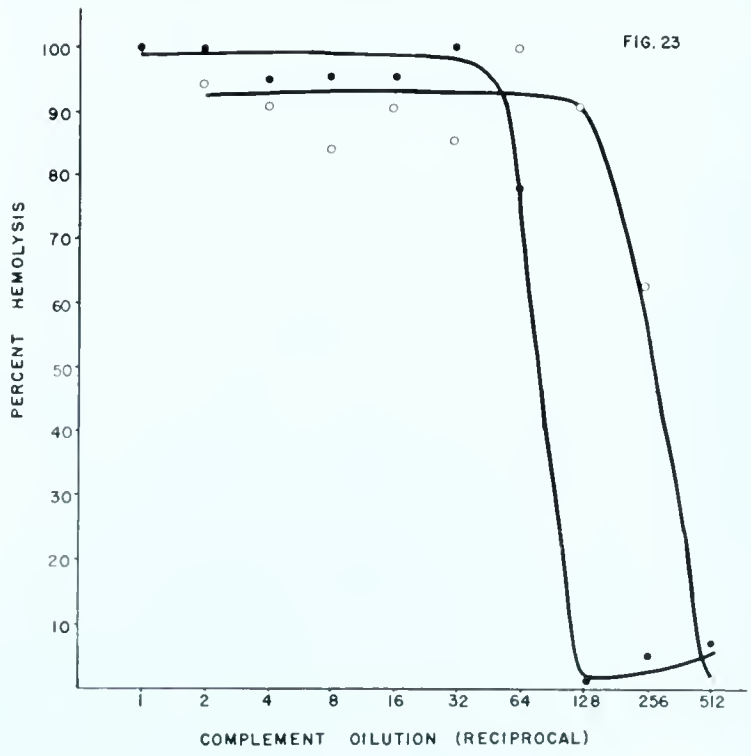

PERCENT HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
1
2
4
8
16
32
64
128
256
512
COMPLEMENT DILUTION (RECIPROCAL)

FIG. 23

D. Comparative Complement Dependence of Indicator and Cytotoxic Isoantibody Systems

The percent hemolysis obtained with 1:1024 hemolysin and with 1:1000 hemolysin plus 1:1024 cytotoxic isoantibody were plotted against complement dilutions in Figure 23. The percent hemolysis for complement dilutions of 1:1 to 1:32 was lower in the cytotoxic-hemolysin system than in the hemolysin system alone. The percent hemolysis at low complement dilutions was slightly lower for the cytotoxic-hemolysin titration (B) than for the hemolysin system (A). This probably reflects complement utilization by the cytotoxic isoantibody. There also may have been some anti-complementary effect of mouse isoantiserum. However, at a dilution of 1:1024, any such anti-complementary effect should be diluted out completely. This phenomenon was not due to inhibition of hemolysis by the isoantiserum, as a titration of C3H anti C57L serum (1:4 to 1:1024), C3H spleen cells, complement and hemolysin resulted in 100% hemolysis. There was slightly more hemolytic antibody present in B than in A and as B also contained cytotoxic isoantibody, the percent hemolysis of B would be expected to fall at a lower dilution. The greater hemolysis in B was not due to complement present on unwashed mouse spleen cells, as washed and unwashed spleen cells titrated with dilutions of hemolysin (1:8 to 1:32768), 1:128 complement and PBS resulted in the same amount of hemolysis at comparable hemolysin dilutions. Nor was the increase in hemolysis due to lysis of sheep red blood cells or mouse red blood cells by the cytotoxic isoantibody, for when hemolysin

was eliminated from both the above titrations, no hemolysis was observed. Rice (98) has reported that mouse complement is not hemolytic to sensitized sheep red blood cells.

Discussion

The measurement of residual complement in the cytotoxic isoantibody titration by the addition of a hemolysin system appeared to measure accurately the extent of complement fixation by the cytotoxic isoantibody-spleen cell isoantigen system. The complement requirements of the two systems were comparable in that at the equivalence point, small differences in complement concentration resulted in large differences in the observable effects of the respective antibodies.

E. Anti-complementary Effects of Mouse Serum

Mouse spleen cells (12,45) and mouse serum, both normal and immune (12,43,101,111,113,124) have all been reported to be inhibitory to complement utilization. Spleen cells used in these experiments were only slightly anti-complementary. As cells were contained in all tubes, their anti-complementary effects would be uniform and correction was not considered necessary in cytotoxic isoantibody titrations where the amount of complement added was uniform. Low dilutions of mouse antiserum were inhibitory to cell death (Figure 3) at 1:8 complement levels, even though there was residual complement present as indicated by hemolysis of sensitized sheep red blood cells (Figure 22). Low dilutions of mouse antiserum and normal mouse serum were inhibitory to

complement fixation in the hemolytic system (Figures 24 and 25). The percent inhibition was calculated by subtracting the percent hemolysis of the antiserum control dilutions (antiserum, complement, PBS, sheep red blood cells and hemolysin) from 100 and adding this correction to the percent hemolysis of the corresponding dilution of antiserum in the test. The results of such a procedure are plotted in Figure 26. In this way, 100% hemolysis was obtained at very high and very low dilutions of mouse antiserum. The 100% hemolysis obtained with low dilutions of antiserum probably reflects the inhibition of complement fixation by excess isoantibody in the cytotoxic titration, thus providing enough residual complement to give 100% hemolysis. This was an unexpected finding, for antibody in the region of antibody excess has been reported to fix complement more readily than antibody in the region where antibody is limiting (8,29). The high percent hemolysis at the highest antiserum dilutions reflects the decreased fixation of complement by a decreased amount of isoantibody. The products of immune lysis have been reported to inhibit complement fixation (95); thus, more complement would be available for the hemolytic system where isoantibody dilution was high.

The central part of the complement fixation curve is believed to represent complement fixation by the cytotoxic isoantibody-isoantigen complexes, for this was the region where maximum cell death was demonstrable (Figure 27). However, the same general type of curve is illustrated in Figure

Figure 24. Complement fixation of normal C3H serum titrated against normal C3H spleen cells in the presence of 1:64 complement. Modified Gorer and O'Gorman method not corrected (●) and corrected (o) for anti-complementary effect of mouse serum.

Figure 25. Complement fixation of C57L anti A serum titrated against normal Strong A spleen cells in the presence of 1:32 complement. A - Terasaki's method. B-Terasaki's method, supernatant readded. C - Supernatant from A. D - modified Gorer and O'Gorman method.

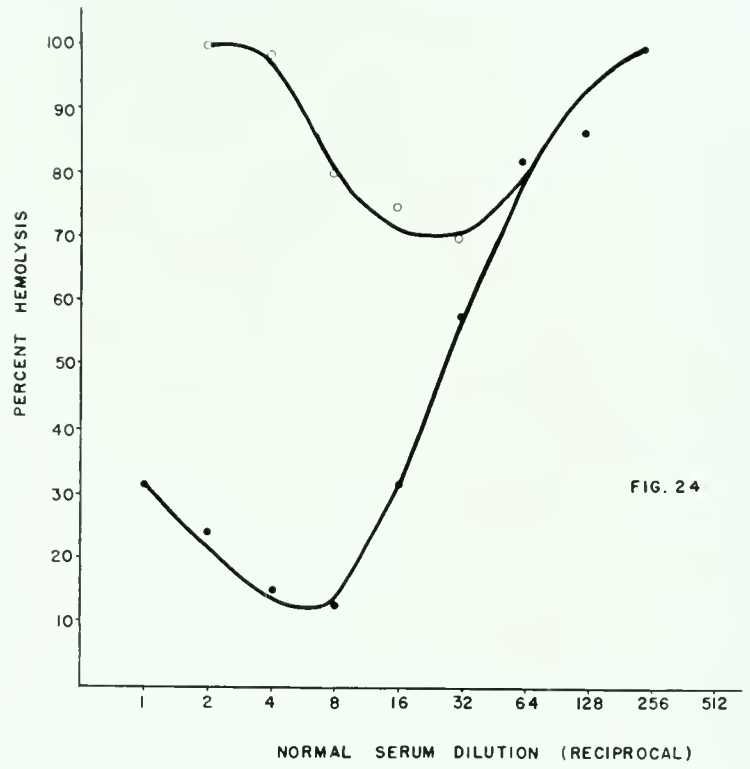

PERCENT HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
1
2
4
8
16
32
64
128
256
512
NORMAL SERUM DILUTION (RECIPROCAL)

FIG. 24

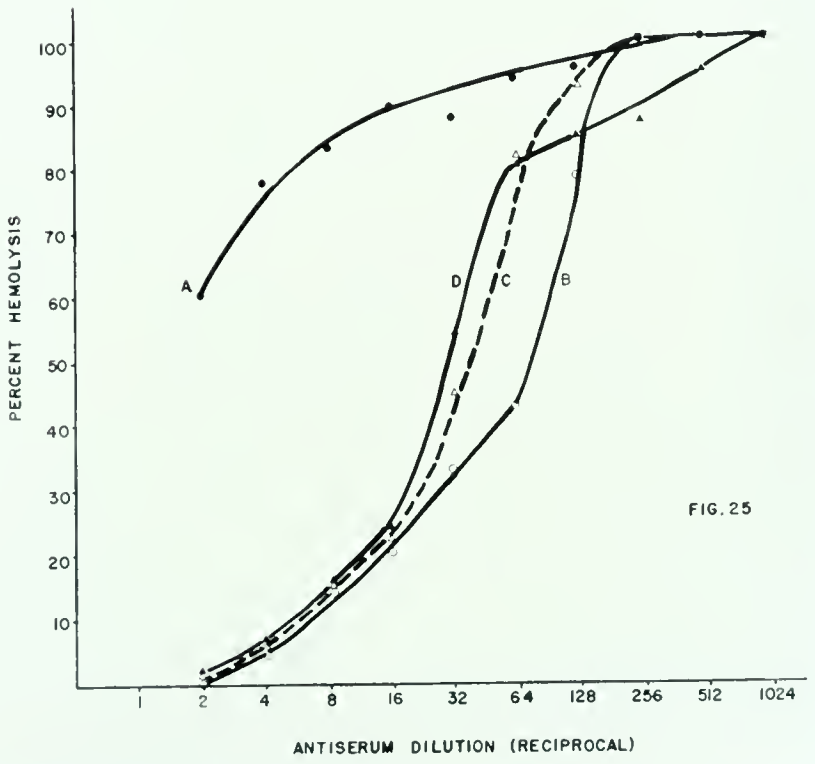

PERCENT HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
A
D
C
B
1
2
4
8
16
32
64
128
256
512
1024
ANTISERUM DILUTION (RECIPROCAL)

FIG. 25

Figure 26. Same data as Figure 25, with B, C, and D corrected for the anti-complementary effects of mouse serum using an anti-serum control.

Figure 27. Percent relative mortalities of Strong A spleen cells incubated with C57L anti A serum in the presence of 1:32 complement. A - Terasaki's method. B - Terasaki's method, supernatant readded. C - Supernatant from A. D - modified Gorer and O'Gorman method.

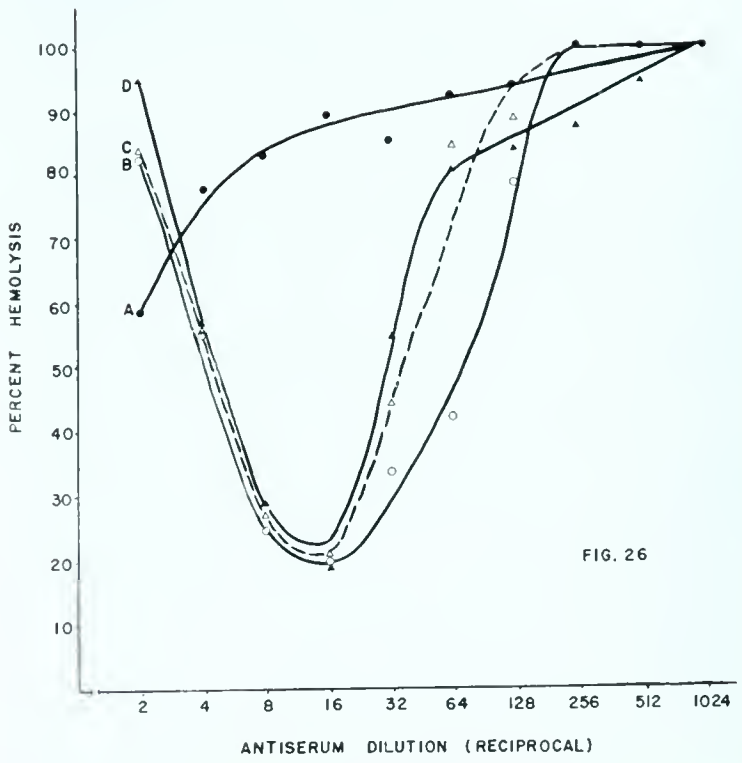

PERCENT HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
D
C
B
A
2
4
8
16
32
64
128
256
512
1024
ANTISERUM DILUTION (RECIPROCAL)

FIG. 26

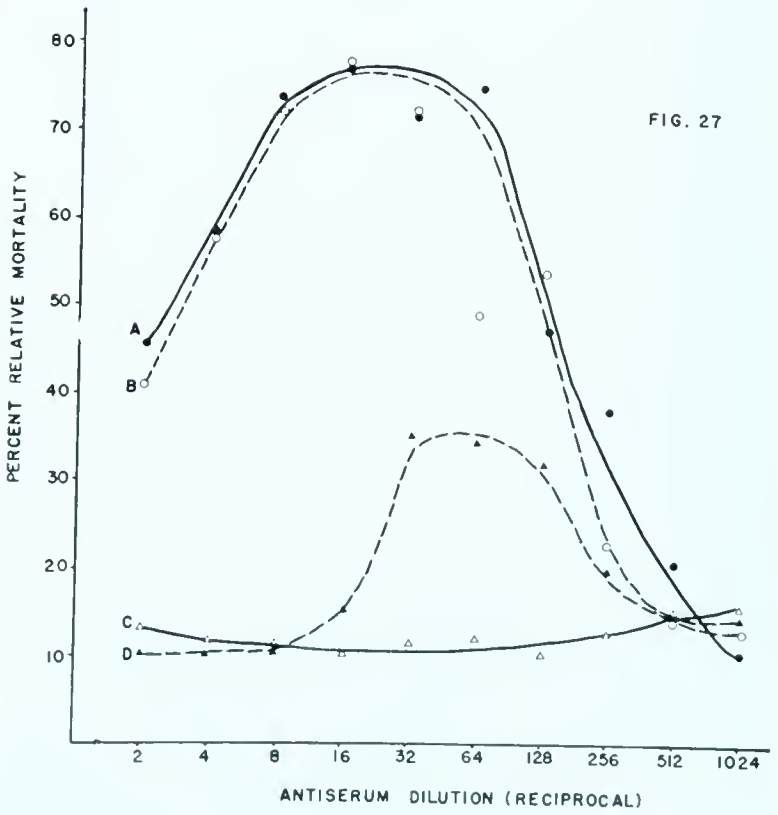

PERCENT RELATIVE MORTALITY
80
70
60
50
40
30
20
10
A
B
C
D
2
4
8
16
32
64
128
256
512
1024
ANTISERUM DILUTION (RECIPROCAL)

FIG. 27

24, where normal serum was titrated against normal mouse cells. In the latter case, the lowered percent hemolysis probably reflects the equivalence zone of the natural guinea pig anti-mouse antibody.

Terasaki (113) attempted to eliminate the anti-complementary action of mouse antiserum by removing the antiserum before the addition of complement. All of the antisera used in these experiments had sufficiently high cytotoxic titers that anti-complementary effects were diluted out completely where maximum cell death was observed. We have investigated the phenomenon of decreased complement fixation by low dilutions of mouse antiserum, using Terasaki's method (113).

Two systems were compared: (a) C57L spleen cells, A anti C57L serum, and 1:32 complement were titrated by the technique described on page 21; (b) the above titration was also carried out using a modification of Terasaki's method (113), whereby the antiserum and spleen cells were incubated for 15 minutes in a water bath at 37°C., centrifuged at 1769 x g for 10 minutes, and the supernatant containing most of the antiserum removed. PBS was added to each tube to restore the volume, complement was added, and the tubes were reincubated for 15 minutes. The hemolysin system (page 26) was then added to both systems. The results of the titration (Figures 25 and 26) illustrate a large discrepancy in the complement utilization of the two systems. Terasaki's method apparently utilizes less complement.

Pikovski (92) described two isoantibodies present in mouse antiserum, a complement dependent isoantibody which reacted against cell surface membranes increasing permeability to dyes, and a complement independent isoantibody which penetrated damaged cells and produced a precipitation of the cytoplasmic antigens. Although the isoantibody which precipitated the cytoplasmic antigens was not dependent on complement for its action, it seems possible that such an isoantibody could fix complement. Using Terasaki's method, isoantibody would be bound to the cell surface membranes, but would not affect the permeability of the membranes until complement was added. Therefore, the complement independent isoantibody would not penetrate the cells and would be removed before complement was added. Even if the complement independent isoantibody were capable of fixing complement, it could not do so in Terasaki's titration, and thus a larger complement residual would be available and 100% hemolysis ensue. This possibility was investigated. Four series of tests were set up; the first and second (A and B) consisted of 0.1 ml. dilutions of C57L anti A serum (1:1 to 1:1024), and 0.1 ml. of normal Strong A cells which were incubated in a 37°C. water bath for 15 minutes. All the tubes were centrifuged at 1769 x g for 10 minutes and the supernatants removed. To each tube was added 0.1 ml. of 1:64 complement and the tubes reincubated for 15 minutes. At the end of the second incubation, A was brought to volume with PBS and the supernatant removed from B was readded to B. The tubes were then reincubated

for 15 minutes. The third series of tests (C) consisted of the supernatants from A, 0.1 ml. of normal Strong A spleen cells and 0.1 ml. of 1:64 complement. The fourth series (D) was the modified Gorer and O'Gorman (46) titration (page 21). Series C and D were incubated for 30 minutes in a 37°C. water bath. The hemolysin system was added to the four test series (page 26) and the percent hemolysis determined as described on page 27. Adequate controls were included (page 26). The four test series were set up in duplicate, the percent relative mortality determined on one set and the percent hemolysis on the other set.

Results and Discussion

Terasaki's method (A) showed a large excess of residual complement (Figures 25 and 26) when compared with the modified Gorer and O'Gorman method (D). This could not be attributed to simple removal of the anti-complementary effect by removing most of the mouse serum before the addition of complement. Comparison of the residual complement in A and D, showed that the residual complement in A was not progressively greater than D, as higher isoantiserum dilutions were approached. Instead, the difference was greatest in the middle zone centered on the zone of maximum cell lethality (Figure 28). This suggested that the difference in complement utilization by the cytotoxic isoantibody in the two methods was due to isoantibody-isoantigen reaction utilizing more complement in the Gorer and O'Gorman system. Thus, Terasaki's method did not measure all the isoantibody-isoantigen reactions that

Figure 28. Percent relative mortality (●) of Strong A cells titrated with C57L anti A serum and 1:32 complement, by Terasaki's method. Percent hemolysis (o) of the sheep red blood cells by the anti sheep hemolysin reflects the excess of unfixed complement in the Terasaki method as compared to that present in the modified Gorer and O'Gorman method. Note that the peak cytotoxic effect corresponds with the maximum complement fixation of the modified Gorer and O'Gorman method.

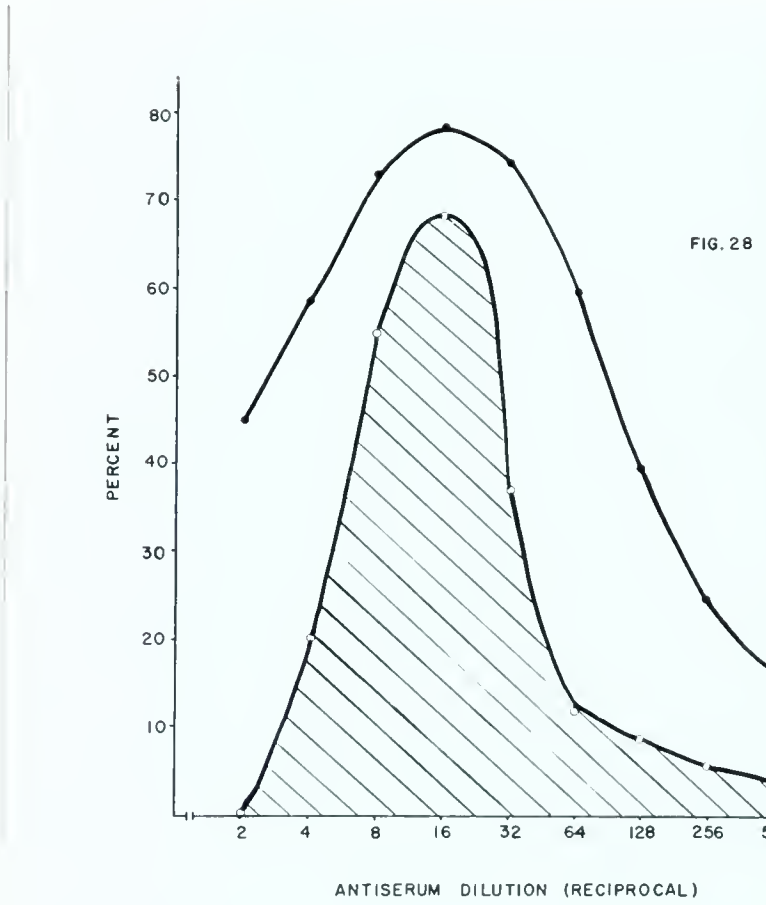

80
70
60
50
40
30
20
10
PERCENT
2
4
8
16
32
64
128
256
512
1024
ANTISERUM DILUTION (RECIPROCAL)

FIG. 28

fix complement. Presumably, isoantibodies capable of fixing complement were removed before the addition of complement in Terasaki's method.

If only one isoantibody were present, B and D should have shown the same percent hemolysis and relative mortality. The percent relative mortalities were drastically different (Figure 27), and a 50% end point was not obtained for D. Competition of the cytotoxic isoantibody and the postulated second isoantibody(s) for the limited complement available, might account for the reduced cell death observed in D. When 1:8 complement was used, the percent relative mortalities of A, B and D were the same. Although the percent hemolysis curves were similar in shape (Figures 25 and 26), B fixed more complement than D at isoantibody dilutions of 1:32 to 1:128. The percent hemolysis of C should have been 100 if the cytotoxic isoantibody had been absorped out by the reaction in A. However, the percent hemolysis was not 100 throughout, and the curve compared well with the percent hemolysis curve of D (Figures 25 and 26). Furthermore, cell death was not observed (Figure 27). (A cytotoxic isoantibody titration of C with 1:8 complement also did not demonstrate cell death.) Therefore, the complement was not utilized by a isoantibody lethal to the target spleen cells. However, the second isoantibody(s) is probably one which reacts on cell surfaces, for complement was utilized in C which contained live spleen cells.

It is concluded that A measured the complement fixation of the cytotoxic isoantibody reaction in the absence of anti-complementary effects of mouse serum. Maximum cell death was obtained with A and did not appear to require the further complement utilization shown in C. B and D therefore measured the complement utilization of both cytotoxic and non-cytotoxic isoantibodies. The mouse antiserum used in these titrations was collected after six immunizing injections, and repeated immunization is generally believed to produce a more efficient, multivalent antibody globulin (95). Presumably, such a multivalent antibody could utilize more complement. However, C, where the percent relative mortality was negligible, could not represent increased complement utilization by a multivalent cytotoxic isoantibody. Therefore, a second non-cytotoxic isoantibody(s) is thought to have been present.

Our control corrections did not result in complement fixation curves like those for Terasaki's method because the complement was more effectively utilized in our system. The complement utilization curves, corrected for anti-complementary effects of mouse serum by the use of a control, appear to have been a proper reflection of complement utilization in the system. The shapes of the curves were determined by a prozone, an equivalence zone, and a postzone in the cytotoxic system.

F. Summary

1. Heterologous complement is necessary to demonstrate the cytotoxic activity of cytotoxic isoantibody. A dilution of 1:8 guinea pig complement provides an adequate excess of active complement. The guinea pig anti-mouse antibody is completely diluted out at this dilution of complement.

2. Complement fixation in the cytotoxic system can be accurately measured with a rabbit hemolysin-sheep red blood cell system.

3. Normal and isoimmune mouse sera are anti-complementary.

4. In Terasaki's method, anti-complementary effects of mouse serum are greatly reduced by removal of most of the antiserum before the addition of complement.

5. More complement is utilized in the modified Gorer and O'Gorman method than in Terasaki's method. Complement fixation in the former method appears to indicate the presence of both a cytotoxic and a non-cytotoxic isoantibody(s).

VIII. EFFECT OF TREATMENT WITH ISOANTISERUM ON THE PRODUCTION OF SPLENIC NODULES BY INTRAVENOUS INJECTION OF ISOGENIC SPLEEN CELLS IN LETHALLY IRRADIATED MICE

Large numbers of isogenic bone marrow or spleen cells, when injected into lethally irradiated mice, will protect the mice from radiation death by repopulation of the spleen and other haemopoietic tissues (116). Smaller numbers of haemopoietic cells, while not protecting the animal from death, produce discrete colonies of proliferating cells in the spleen (115,116). It is not known which cell types are capable of multiplication when transplanted into lethally irradiated mice; however, it is unlikely that fully differentiated cells would have this capacity (70). The effective cells must be present as a constant proportion of the total numbers of nucleated cells, as there is a linear relationship between the number of colonies formed and the cell dose (70,71). Colony counts of greater than 15 per spleen cease to be linear (71).

A. Pilot Experiment

An isogenic system (C3H → C3H) was studied to determine the cell dose necessary to produce splenic nodules and to investigate the relationship between cell dose and number of nodules produced.

Twelve C3H male mice four months old were subjected to whole body irradiation. A dose of 89.3 roentgens per minute was administered 2.5 cm. beneath the surface of a perforated 20 x 20 cm. plexiglass irradiating cage with a Picker x-ray machine (260 Kv, 16Ma, 0.5 mm. Cu filter). The mice received a total dose of 902 roentgens. After irradiation, the mice were divided into four comparable groups on the basis of body weight.

Two C3H male mice, four months old, were sacrificed and a spleen suspension prepared as described on page 16. Dilutions containing approximately 10^7, 10^6, 10^5 and 10^4 cells per 0.5 ml. were made of this suspension. Each mouse of each group received 0.5 ml. of the appropriate cell dilution intravenously. The spleen cell suspensions were kept in an ice-water bath during the injection procedure. Each cell dilution was examined for stained cells with 1.5% lissamine green. Table XII illustrates the cell dosage received by each group.

All surviving mice were sacrificed 10 days after the spleen cell injections. Body, spleen, pooled inguinal and axillary lymph nodes, thymus and mesenteric lymph nodes were weighed. These weights were then converted to mgm. per 100 grams of the groups' mean autopsy body weight (Table XII). The visible nodules on each spleen were counted.

Discussion

The above experiment confirmed the observation of Till and McCulloch (115) and Trentin and Fahlberg (116) that

Table XII

Organ Weights and Macroscopic Nodules Present on Spleens of Lethally Irradiated C3H mice Receiving Various Doses of Isogenic Spleen Cells

Group	No. Spleen Cells Injected ($\times 10^4$)			N*	Autopsy Body Wt. (gms.)	Mgm/100 gm. Autopsy Body Wt.				
	Total	Unstained	Stained			Spleen	Lymph Nodes	Mesenteric Nodes	Thymus	Macroscopic Nodules
A	980	850	130	2/3	23.0	205	19	99	53	7
B	98	58	40	2/3	19.6	120	41	113	17	1.5
C	20	16	4	2/3	22.6	130	54	102	18	2
D	2	2	0	1/3	22.9	103	48	117	17	0

*N = $\frac{\text{No. of survivors}}{\text{No. injected}}$

splenic nodules can be produced by injecting live isogenic spleen cells into lethally irradiated mice. Furthermore, there was a direct relationship between the number of cells injected and the number of visible nodules produced in the spleen (Figure 29). Till and McCulloch (115) state that 10^4 cells are needed to produce one visible nodule. Since the spleen constitutes approximately 1/200 of the total body volume, of an injection dose of 10^4 cells, 50 cells would reach the spleen. Consequently, it would appear that 50 cells must reach the spleen to result in the appearance of a single nodule. When the numbers of colonies per spleen were plotted against the numbers of cells injected, the line went through the origin. It would appear then, that a single viable cell of the appropriate type, upon reaching the spleen, could give rise to a colony (115). Trentin calculated that of every 32 cells which reach the spleen, only one will implant and produce a nodule. Our experiment indicated that approximately 5×10^4 cells were necessary to produce one nodule, a result of the same order of magnitude as the results obtained by Till and McCulloch (115), and Trentin and Fahlberg (116).

B. Effect of Cytotoxic Antiserum on the Nodule-Forming Capacity of Isogenic Spleen Cells in Lethally Irradiated Recipients

The cytotoxic isoantibody titration method of Gorer and O'Gorman (46) presumably measures all the cells killed by the action of cytotoxic isoantibody. If this assumption

is valid, treatment of spleen cell suspensions with increasing dilutions of specific isoantibody would result in a series of cell suspensions containing increasing numbers of live cells, and these cells should be capable of producing colonies in the spleens of lethally irradiated mice. This hypothesis was tested in the following experiment.

Forty-eight male C3H mice three months old were exposed to whole body irradiation in groups of twelve in perforated (20 cm. x 20 cm. x 5 cm.) plexiglass cages. A dose of 89.3 roentgens per minute was administered by a Picker x-ray machine (260 Kv 16 Ma with a 0.5 mm. copper filter) at 2.5 cm. beneath the surface of the cage for a total dose of 800 roentgens.

A normal C3H male mouse three months old was sacrificed and a spleen cell suspension prepared as described on page 16. C57L and C3H serum having a $\log_2$ titer of 5.8 was incubated at 37°C. for one hour in a water bath. To 2.2 cc. each of the undiluted antiserum, 1:128 antiserum and undiluted normal C3H serum, 2.2 ml. of the C3H spleen cell suspension (1.4×10^7 cells/ml.) was added in siliconized test tubes. The tubes were corked and incubated for 15 minutes at 37°C. in a water bath. After incubation, 4.2 ml. was removed from each tube and kept in siliconized tubes in an ice bath during the injection procedure. To the remaining 0.2 ml. in each of the tubes, 0.1 ml. of 1:8 guinea pig complement was added and then incubated for a further 15 minutes. The proportion of stained cells was determined as described on page 21.

Each of the 12 mice in Group I received 0.3 ml. of the spleen cell suspension incubated with 1:1 antiserum. This suspension contained 49.4% stained cells and therefore each mouse received 1.1×10^6 unstained and presumably live cells. Group II received 0.3 ml. of the spleen cell suspension incubated with 1:128 antiserum. This suspension contained 12.1% stained cells and therefore each mouse received 1.9×10^6 live cells. Group III mice each received 2.1×10^6 live cells as they were injected with the spleen cell suspension incubated with normal serum and which contained 1.4% stained cells. Group IV did not receive any cells. All the injections were done intravenously within a one-hour period, one day after the radiation was administered. All the surviving mice were sacrificed 10 days after the spleen cell injections. Body weight, spleen, thymus, mesenteric node and pooled axillary and inguinal lymph node weights were determined at autopsy. Organ weights were converted to mgm. per 100 grams autopsy body weight (Table XIII) and macroscopically visible nodules on the spleens were counted.

Discussion

As all the live cell doses were in the range of 10^6 spleen cells per mouse, it might have been expected that the number of visible splenic nodules in each group would be about the same. Such was not the case (Figure 30). The group receiving the highest concentration of antiserum (Group I) produced the smallest number of nodules. However, the group

Table XIII

Organ Weights and Macroscopic Nodules Present in Spleens of Lethally Irradiated C3H Mice Receiving a Constant Dose of Isogenic Spleen Cells Treated with Varying Doses of C57L anti C3H Serum

Group	Cell Dose ($\times 10^6$)			N*	Original Body Wt. (gms.)	Autopsy Body Wt. (gms.)	Mgm./100 gm. Autopsy Body Wt.				Macroscopic Spleen Nodules
	Total	Unstained	Stained				Spleen	Thymus	Mesenteric Nodes	Lymph Nodes	
IV	0	0	0	3/12	20.2	17.2	184.9	85.5	130.8	74.4	0
I	2.2	1.1	1.1	8/12	20.4	14.7	160.5	57.2	108.8	71.6	2
II	2.2	1.9	0.4	8/12	20.2	16.2	237.1	84.0	167.9	86.4	7.5
III	2.2	2.1	0.1	5/12	20.2	19.4	344.9	81.4	141.3	85.1	12.0

*N = $\frac{\text{Number of Survivors}}{\text{Number of Mice Injected}}$

Figure 29. Relationship between spleen cell dose and spleen weight (●) and between cell dose and number of macroscopic splenic nodules (o) in C3H mice exposed to lethal whole body irradiation and then injected intravenously with C3H spleen cells.

Figure 30. Effect of cytotoxic isoantiserum on the transplantability of isogenic spleen cells. There is a marked increase in spleen weight (o) and number of macroscopic splenic nodules (●) with increasing numbers of unstained cells as determined in the cytotoxic isoantibody titration.

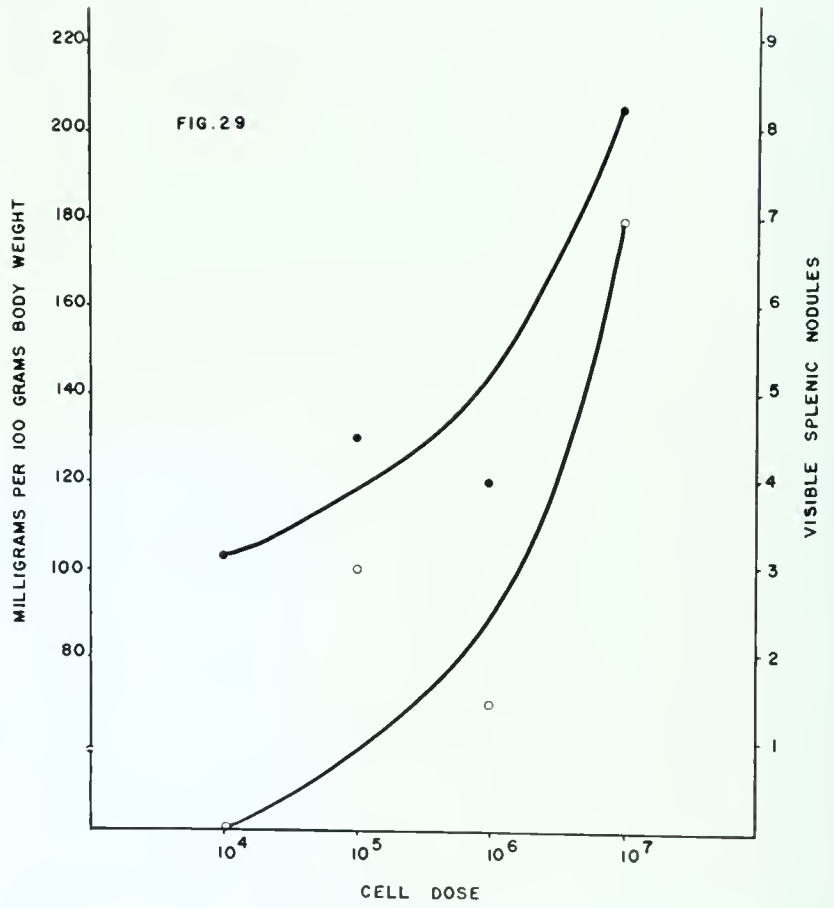


FIG. 29
MILLIGRAMS PER 100 GRAMS BODY WEIGHT
220
200
180
160
140
120
100
80
VISIBLE SPLENIC NODULES
9
8
7
6
5
4
3
2
1
10^4
10^5
10^6
10^7
CELL DOSE

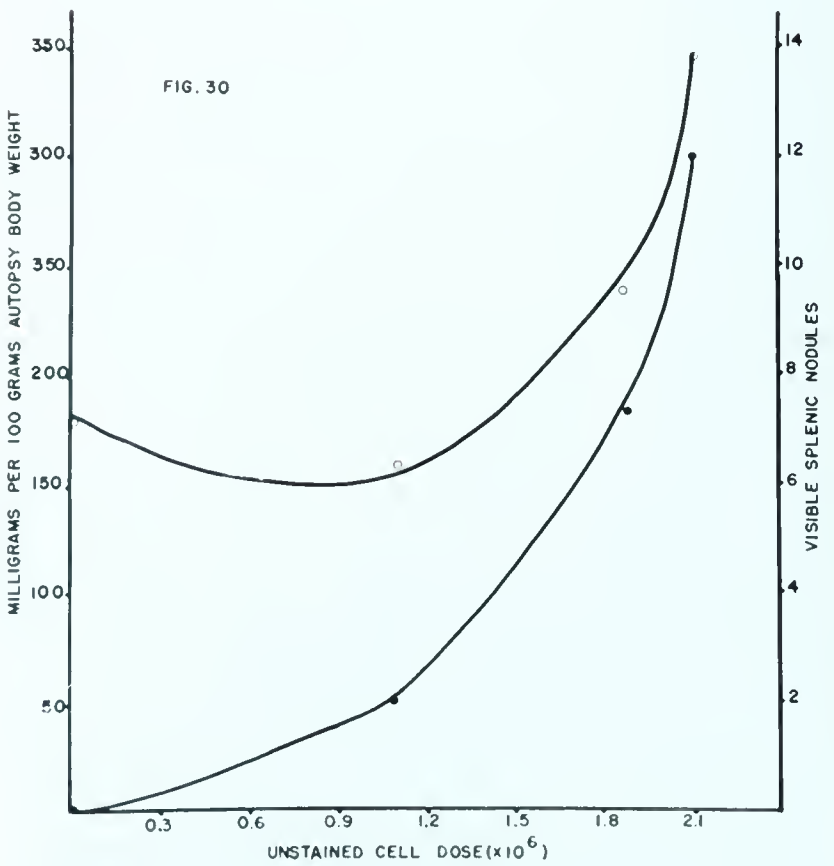


FIG. 30
MILLIGRAMS PER 100 GRAMS AUTOPSY BODY WEIGHT
350
300
350
200
150
100
50
VISIBLE SPLENIC NODULES
14
12
10
8
6
4
2
0.3
0.6
0.9
1.2
1.5
1.8
2.1
UNSTAINED CELL DOSE($\times 10^6$)

receiving the highest dilution of antiserum (Group II) produced almost four times as many nodules, and the group receiving normal serum (Group III) produced six times as many nodules as Group I. It would appear then, that the unstained cells of Group I and Group II were not necessarily alive, or if alive were incapable of implantation and proliferation. If all the unstained cells were capable of producing colonies, 9.6 nodules would have been expected in Group II and 6.0 nodules in Group I.

C. Effect of Cell Dose on Production of Splenic Nodules in Lethally Irradiated Isogenic Recipients

Eighty-eight female C3H mice 7 to 9 months old were given whole body irradiation as described on page 105. A dose of 71.7 roentgens per minute was delivered, giving a total dose of 799 roentgens. The mice were then divided on the basis of body weight into eight comparable groups of 11 mice each. Groups I to IV received a constant number of spleen cells treated with various dilutions of antiserum. Groups V to VIII received varying numbers of spleen cells.

1. Constant Cell Numbers with Serial Dilutions of Antiserum

A normal male C3H mouse 8 months old was sacrificed and a spleen suspension prepared as described on page 16. C57L anti C3H serum (day 82) with a $\log_2$ titer of 7.0 was diluted 1:8, 1:16, 1:64 and 1:128. Five ml. aliquots of the C3H spleen cell suspension was incubated with 5 ml. of each of the antiserum dilutions in siliconized test tubes. Incubation was at 37°C. in a water bath for 15 minutes. After

incubation, 0.2 ml. of each of the tubes was removed, added to 0.1 ml. of 1:8 guinea pig complement and reincubated for 15 minutes. The proportion of stained cells was determined by the method described on page 21. The number of live cells remaining in each tube was calculated. Each mouse received 0.4 ml. of the respective spleen cell-antiserum dilution mixtures intravenously. The cell dosage received by each group is shown in Table XIV. All surviving mice were sacrificed 10 days after the spleen cell injections. Body weight, spleen, thymus, mesenteric lymph node, and pooled axillary and inguinal lymph node weights were determined at autopsy. Organ weights were converted to mgm. per 100 gram autopsy body weight. The spleens were numbered and allowed to stand in formalin for two weeks. This treatment facilitates nodule counting for the nodules shrink less than the spleen does and are thus more prominent. The numbers of red and white nodules were recorded separately (Table XIV).

2. Serial Dilution of Cells in Phosphate-Buffered Saline

A normal male C3H mouse 8 months old was sacrificed and a spleen suspension prepared as described on page 16. Dilutions of this spleen suspension were made to correspond roughly with the number of unstained cells administered in Part One of the experiment. Each mouse of each group received 0.4 ml. of the respective cell dilution intravenously.

All surviving mice were sacrificed 10 days after the spleen cell injections. The organ weight and nodule counts

Table XIV

Comparison of the Nodule-Forming Capacity of Antiserum Treated Spleen Cells and Various Dilutions of Untreated Spleen Cells

Group	N*	Cell Dose ($\times 10^6$)			Body Weight (gms.)		Mgm/100 gms. Autopsy Body Wt.				No. Macroscopic Nodules		
		Unstained	Stained	Total	Original	Autopsy	Spleen	Thymus	Mes. Nodes	Pooled Lymph Nodes	Red	White	Total
II	10/11	0.78	0.77	1.55	26.1	24.2	145.3	86.0	97.5	72.4	1.1	1.1	2.2
I	6/10	0.96	0.59	1.55	25.9	23.5	130.0	62.8	95.6	63.7	1.6	0.9	2.5
III	11/11	1.23	0.32	1.55	26.1	22.7	138.7	109.6	110.8	69.0	1.2	1.1	2.3
IV	11/11	1.38	0.17	1.55	26.2	22.2	151.2	102.8	110.6	78.6	2.5	2.6	5.2
VIII	11/11	-	-	0.60	26.4	24.2	166.9	117.3	107.3	73.3	4.3	3.1	7.4
V	11/11	-	-	1.12	26.0	24.0	174.5	154.5	101.1	73.7	4.0	3.6	7.6
VII	11/11	-	-	1.24	26.1	24.9	210.1	101.4	109.1	70.7	4.8	6.6	11.4
VI	11/11	-	-	1.70	25.9	24.2	214.4	104.5	93.4	79.8	5.2	9.6	14.8

*N = $\frac{\text{Number of Survivors}}{\text{Number of Mice Injected}}$

were performed in the same way as those of Part One. Table XIV illustrates the cell dosage, organ weights (mgm./100 gm. autopsy body weight) and numbers of visible nodules.

3. Discussion

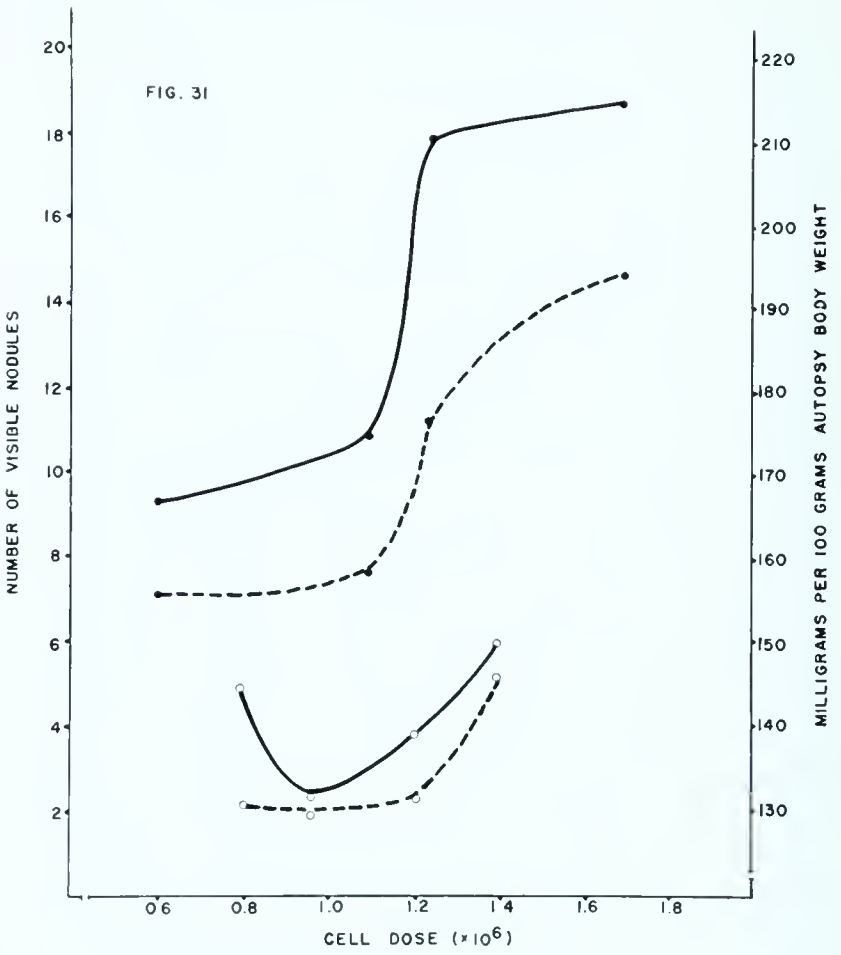
The above experiments illustrated that there was a difference between the nodule producing capacity of antiserum treated and untreated spleen cells (Figure 31). It appeared that antiserum inhibited the production of spleen nodules. One explanation for this inhibition could be that all the unstained cells in the antiserum treated preparation were not viable, and thus were unable to produce splenic colonies. We have shown (page 35) that the cells of the spleen are differentially susceptible to the action of cytotoxic iso-antibody, the small cells being the most resistant and the medium-sized cells the most susceptible. McCulloch and Till (70) state that the cells which are capable of proliferation and nodule formation in the spleens of lethally irradiated mice must be evenly distributed and a constant proportion of the nucleated cells. Perhaps these effective cells are the medium-sized cells which are preferentially killed by the isoantibody. Thus even though the antiserum treated spleen cell suspension contained numerous live cells, many of these cells might have been incapable of multiplication and proliferation.

D. Clonal Nature of Splenic Nodules

Becker, McCulloch and Till (13) have attempted to demonstrate the clonal nature of spleen colonies produced

Figure 31. Spleen weight (-) and number of macroscopic splenic nodules (---) in C3H mice exposed to lethal whole body irradiation and injected intravenously with either freshly prepared isogenic spleen cells (●), or spleen cells suspension treated with C57L anti C3H serum (o). Cell doses are plotted as number of unstained cells. Note difference in spleen weight and number of nodules in recipients of fresh as compared with antibody treated spleen cells.

FIG. 31


NUMBER OF VISIBLE NODULES
MILLIGRAMS PER 100 GRAMS AUTOPSY BODY WEIGHT
CELL DOSE (×10⁶)
20
18
16
14
12
10
8
6
4
2
220
210
200
190
180
170
160
150
140
130
0.6
0.8
1.0
1.2
1.4
1.6
1.8

by transplanting haemopoietic cells into lethally irradiated recipients. Although they have found that within a given colony, cells of the erythrocytic, granulocytic and megakaryocytic series may be present, they believe that each colony is a clone. Their belief that each colony was formed from a single cell is supported by the observation that the curves relating the number of cells transplanted to the number of colonies formed is linear and does not show an initial threshold. Furthermore, by the use of irradiated donor marrow cells which provide some cells with abnormal karyotypic mitoses, they have shown in 8 colonies that 73-95% of the cells in metaphase in each colony had the same mitotic abnormality.

If the splenic nodules were clonal, the cells of the clone should be susceptible to a cytotoxic antiserum specificity directed against the H-2 antigens of the cells. Furthermore, the cells should not be susceptible to an antiserum directed against H-2 antigens not present on the cell. The following experiments were an attempt to validate the clonal nature of splenic colonies produced in lethally irradiated mice.

Twenty male Strong A mice (Groups A and C) and twenty male LAF_1 mice (Groups B and D) 3 months old were subjected to whole body irradiation as described on page 105. A dose of 71.7 roentgens per minute was administered to Groups A and B, resulting in a total dose of 799 roentgens. Groups C and D received 800 roentgens administered at 73.0 roentgens per minute. A normal male Strong A mouse 3 months old and a

normal male C57L mouse 4 months old were sacrificed and their spleens suspended as described on page 16. The spleen cell suspensions were then diluted so each contained approximately 2.5×10^5 cells/0.5 ml. Equal volumes of the Strong A and the C57L spleen cell suspensions were made. Each of the mice of Groups A and B received 0.5 ml. intravenously of a spleen suspension containing 2.6×10^5 Strong A cells/0.5 ml. and 2.8×10^5 C57L cells/0.5 ml. Each mouse in Groups C and D received 0.4 ml. of a spleen cell suspension containing 3.5×10^5 Strong A cells/0.4 ml. and 3.8×10^5 C57L cells/0.4 ml. All the surviving mice were sacrificed 10 days after the spleen cell injections and the spleens were examined for visible nodules. All nodules which were sufficiently large and well-separated were carefully dissected with a sharp razor blade. The nodules were each suspended in 0.3 ml. of PBS with a sterile needle. Each nodule was titrated with C57L anti A and A anti C57L serum. In addition, normal C57L, normal Strong A and normal LAF_1 spleen cells were titrated with normal sera and with both antisera. Both antisera were used at a dilution which resulted in 70 to 95% stained cells with their specific target cells. Both antisera were 80 to 90% lethal to LAF_1. The results of this experiment are shown in Table XV, where the percent stained cells observed are recorded for each antiserum. Suspending the nodules with a needle is more traumatic than suspending with a screen; therefore, 20% stained cells are accepted as the number of cells which could have been injured during preparation.

Table XV

Histogenetic Analysis, Using Specific Cytotoxic Antisera, of Splenic Nodules Produced in Lethally Irradiated Mice Injected with a Mixture of Strong A and C57L Spleen Cells

Antiserum	% Stained Cells			
	Group A*	Group B	Group C	Group D
C57L anti A				
1	89.0	57.5	79.0	53.7
2	89.3		97.9	90.4
3	81.3		25.2	65.3
4	92.7		83.4	95.5
5	55.9		64.2	
6	76.5		86.9	
7			72.2	
8			86.4	
A anti C57L				
1	55.7	17.1	7.5	46.8
2	21.2		9.6	41.6
3	18.7		5.5	15.0
4	12.4		50.0	64.6
5	24.8		12.6	
6	13.3		1.7	
7			32.3	
8			21.1	

*Groups A and C = Strong A mice.
Groups B and D = LAF_1 mice.

Discussion

It appears that most of the nodules dissected from the spleens of Strong A recipients, developed from Strong A donor cells. Group A nodule number 2, 3, 4 and 6 and Group C nodule number 1, 2, 5 and 6 were predominantly comprised of cells which were sensitive to C57L anti A serum. Group C nodules 7 and 8 probably were also Strong A cells. However, Group A nodules 1 and 5 and Group C nodules 3 and 4 appeared to be of mixed composition as these cells were partially sensitive to both antisera. These nodules may, however, have been composed of C57L cells and contaminating Strong A cells, if the colony had not been cleanly dissected.

Fewer colonies were found in the spleens of LAF_1 recipients, although very few mice died before the end of the experiment. McCulloch and Till (72) also found colony repression when parental strain cells were injected into lethally irradiated (C57BL x C3H)F_1 mice. They found that a large proportion of the animals survived until the time of sacrifice, even though some of the spleens were devoid of colonies. Only a few nodules were large enough to be dissected from the LAF_1 recipient spleens. Of these, only Group D nodule 3 was demonstrable as being of one cell type (Strong A). The remaining colonies were composed of cells which were sensitive to both antisera.

If these colonies were truly clonal, they should have been composed of a cell type which was sensitive to

only one antiserum. This was true of most of the colonies dissected from Strong A recipient spleens. However, all except one of the colonies isolated from LAF_1 recipients and four colonies isolated from Strong A recipient spleens were sensitive to both antisera. The colonies may have been partially contaminated with host cells if the dissection was not clean. It is doubtful, however, that 50-80% of the cells could have resulted from contamination. Failure to demonstrate the clonal nature of the colonies could be due to the fact that the colonies were not clonal in nature but were formed from two or more cells. Also, as only the very large nodules were dissected, these colonies could be a collection of two or more coalescent colonies.

E. Summary

1. Splenic nodules can be produced by the injection of isogenic spleen cells into lethally irradiated C3H mice.

2. There is a direct relationship between the number of spleen cells injected and the number of splenic nodules produced.

3. Spleen cells which have been incubated with specific cytotoxic antiserum lose some of their capacity to produce splenic colonies.

4. A 50% mixture of cells from the two parental strains had a decreased ability to form splenic nodules in F_1 mice as compared to isogenic mice.

5. Eleven of the 19 splenic colonies tested with specific cytotoxic antisera appeared to be clonal.

BIBLIOGRAPHY

1. Angervall, L. and Carlström, E., On organ weight analysis in experimental animals. Acta Path. Microbiol. Scand., LI, Suppl. 144, 153-155, 1961.

2. Algire, G. H., Weaver, J. M. and Prehn, R. T., Studies on tissue homotransplantation in mice using diffusion chamber methods. Ann. N. Y. Acad. Sci., LXIV, Art. 2, 1009-1013, 1957.

3. Amos, D. B., The agglutination of mouse leukocytes by isoimmune sera. Br. J. Exp. Path., XXXIV, 464-470, 1953.

4. Amos, D. B., Some isoantigenic systems of the mouse. Proc. Third Can. Cancer Conf., 241-258, 1958.

5. Amos, D. B., Possible relationships between the cytotoxic effects of isoantibodies and host cell function. Ann. N. Y. Acad. Sci., LXXXVII, Art. 1, 273-292, 1960.

6. Amos, D. B., Isoantigens of mouse red cells. Ann. N. Y. Acad. Sci., XCVII, No. 1, 69-82, 1962.

7. Amos, D. B. et al., An antibody response to skin homografts in mice. Br. J. Exp. Path., XXXV, 203-208, 1954.

8. Amos, D. B. and Day, E. D., Passive immunity against four mouse leukoses by means of isoimmune sera. Ann. N. Y. Acad. Sci., LXIV, Art. 5, 851-858, 1957.

9. Amos, D. B., Gorer, P. A. and Mikulska, Z. B., An analysis of an antigenic system in the mouse (the H-2 system). Proc. Royal Soc. London B, CXLIV, 369-380, 1955-56.

10. Amos, D. B. and Wakefield, J. D., Growth of mouse ascites tumor cells in diffusion chambers. II. Lysis and growth inhibition by diffusible isoantibody. J. Nat. Cancer Inst., XXII, 1077-1092, 1959.

11. Basch, R. S. and Stetson, C. A., Quantitative studies on histocompatibility antigens of the mouse. Transpl., I, No. 4, 469-480, 1963.

12. Batchelor, J. R., Complement fixing isoantibodies in mice. Immunol., III, 174-178, 1960.

13. Becker, A. J., McCulloch, E. A. and Till, J. E., Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature, CXCVII, No. 4866, 452-454, 1963.

14. Bennett, B., Old, L. J. and Boyse, E. A., Opsonization of cells by isoantibody in vitro. Nature, CXCVIII, 10-12, 1963.

15. Bitensky, L., Cytotoxic action of antibodies. Br. Med. Bull., XIX, No. 3, 241-244, 1963.

16. Boyd, W. C., Fundamentals of Immunology, 3rd ed., 1956, Interscience Publishers Inc., New York, pp. 361-364.

17. Boyse, E. A., The fate of mouse spleen cells transplanted into homologous and F_1 hybrid hosts. Immunol., II, 170-181, 1959.

18. Boyse, E. A., Old, L. J. and Stockert, E., Immunological enhancement of a leukemia. Nature, CXCIV, 1142-1144, 1962.

19. Boyse, E. A., Old, L. J. and Stockert, E., Some further data on cytotoxic isoantibodies in the mouse. Ann. N. Y. Acad. Sci., XCIX, Art. 3, 575-587, 1962.

20. Brent, L., Medawar, P. B. and Ruszkiewicz, M., Serological methods in the study of transplantation antigens. Br. J. Exp. Path., XLII, 464-477, 1961.

21. Burnet, Sir Macfarlane, The clonal selection theory of acquired immunity. 1959, Vanderbilt University Press, Nashville, Tenn., pp. 30-81.

22. Cann, H. M. and Herzenberg, L. A., In vitro studies of mammalian somatic cell variations. II. Isoimmune cytotoxicity with a cultured mouse lymphoma and selection of resistant variants. J. Exp. Med., CXVII, No. 2, 267-284, 1963.

23. Chouroulinkov, I., Boyse, E. A. and Old, L. J., Cytotoxic activity of isoantibody on sarcoma I. Proc. Soc. Exp. Biol. Med., III, 263-265, 1962.

24. Congdon, C. C., Effect of injection of foreign bone marrow on the lymphatic tissue of normal mice. J. Nat. Cancer Inst., XXVIII, No. 2, 305-329, 1962.

25. Congdon, C. C. and Makinodan, T., Splenic white pulp alterations after antigen injection: Relation to time of serum antibody production. Amer. J. Path., XXXIX, No. 6, 697-709, 1961.

26. Davidsohn, I. and Stern, K., Heterohemo - antibodies in inbred strains of mice. II. Immune agglutination and hemolysis for sheep and chicken red blood cells. J. Immunol., LXXII, 216-223, 1954.

27. Davis, M. L., Cosgrove, G. E. and Gude, W. D., Liver changes in sublethally irradiated mice undergoing the foreign spleen reactions. Radiation Res., XVII, No. 4, 133-139, 1962.

28. Day, E. D. and Amos, D. B., Fractionation and radio-iodination of mouse antiserum globulins. Ann. N. Y. Acad. Sci., CI, Art. 1, 148-159, 1962.

29. Fagraeus, A., Nomenclature of immunologically competent cells. Cellular Aspects of Immunity, 1960, J. A. Churchill, Ltd., London, pp. 3-4.

30. Feldman, M. and Globerson, A., Studies on the mechanism of immunological enhancement of tumor grafts. J. Nat. Cancer Inst., XXV, 631-648, 1960.

31. Feldman, M. and Sacks, L., The antibody response to successful tumor homografts in mice. J. Nat. Cancer Inst., XVIII, 529-553, 1957.

32. Feldman, M. and Yaffe, D., Immunogenetic studies on x-irradiated mice treated with hematopoietic cells and grafted with tumor tissues. J. Nat. Cancer Inst., XXIII, No. 1, 109-131, 1959.

33. Fox, M., Cytological estimation of proliferating donor cells during graft-versus-host disease in F_1 hybrid mice injected with parental spleen cells. Immunol., V, No. 4, 489-495, 1962.

34. Frommhagen, L. H. and Fudenberg, H., The role of aggregated gamma globulins in the anti-complementary activity of human and animal sera. J. Immunol., LXXXIX, No. 3, 336-343, 1962.

35. Gabriel, E. R., Reticular-endothelial activity during antibody response. Ann. N. Y. Acad. Sci., LIX, Art. 3, 337-342, 1955.

36. Gerlings-Petersen, B. T. and Pondman, K. W., Erythro-phagocytosis: A study of the antigen-antibody-complement reaction. Vox. Sang., VII, No. 6, 655-674, 1962.

37. Goldberg, B. and Green, H., The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. I. Ultra-structural studies. J. Exp. Med., CIX, 505-510, 1959.

38. Gorer, P. A., The detection of antigenic differences in mouse erythrocytes by the employment of immune sera. Br. J. Exp. Path., XVII, 42-50, 1936.

39. Gorer, P. A., The genetic and antigenic basis of tumor transplantation. J. Path. Bact., XLIV, 691-697, 1937.

40. Gorer, P. A., The antigenic basis of tumor transplantation. J. Path. Bact., XLVII, 231-252, 1938.

41. Gorer, P. A., Antibody response to tumor inoculation in mice with special reference to partial antibodies. Cancer Research, VII, 634-641, 1947.

42. Gorer, P. A., The antibody response to skin homografts in mice. Ann. N. Y. Acad. Sci., LIX, Art. 3, 365-373, 1955.

43. Gorer, P. A., Some reactions of H-2 antibodies in vitro and in vivo. Ann. N. Y. Acad. Sci., LXXIII, Art. 3, 707-721, 1958.

44. Gorer, P. A., Some recent data on the H-2 system of mice. Biological Problems of Grafting, 1959, C. C. Thomas, pp. 25-33.

45. Gorer, P. A. and Boyse, E. A., Some reactions observed with transplanted reticulo-endothelial cells in mice. Les Congrès et Colloques de L'Université de Liège, Biological Problems in Grafting, XII, 1959, 193-204.

46. Gorer, P. A. and O'Gorman, P., The cytotoxic activity of isoantibodies in mice. Transpl. Bull., III, 142-143, 1956.

47. Gorer, P. A. and Mikulska, Z. B., The antibody response to tumor inoculation. Improved methods of antibody detection. Cancer Research, XIV, 651-655, 1954.

48. Gorer, P. A. and Mikulska, Z. B., Some further data on the H-2 system of antigens. Proc. Royal Soc., London, B, CLI, 57-69, 1959.

49. Gorer, P. A., Mikulska, Z. B. and O'Gorman, P., The time of appearance of isoantibodies during the homograft response to mouse tumors. Immunol., II, 211-218, 1959.

50. Gowans, J. L., Gesner, B. M. and McGregor, D. D., The immunological activity of lymphocytes. Biological Activity of the Leucocyte, 1961, J. and A. Churchill, Ltd., London, pp. 32-44.

51. Green, H., Fleicher, R. A., Barrow, P. and Goldberg, B., The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. II. Chemical studies, J. Exp. Med., CIX, 511-521, 1959.

52. Green, H. and Goldberg, B., The action of antibody and complement on mammalian cells. Ann. N. Y. Acad. Sci., LXXXVII, Art. 1, 352-362, 1960.

53. Halbert, S., Experimental Autosensitization by Lens, Hereditary, Developmental and Immunologic Aspects of Kidney Disease, ed. J. Metcoff, XIII, 1961, pp. 77-78.

54. Hanks, J. H. and Wallace, J. H., Determination of cell viability. Proc. Soc. Exp. Med. Biol., XCVIII, 188-192, 1958.

55. Hildemann, W. H. and Medawar, P. B., Relationship between skin transplantation immunity and the formation of humoral isoantibodies in mice, Immunol., II, 44-52, 1959.

56. Holmberg, B., On permeability to lissamine green and other dyes in the course of cell injury and cell death, Exp. Cell Res., XXII, 406-414, 1961.

57. Ishizaka, K., Ishizaka, T. and Campbell, D. H., Biologic activity of soluble antigen-antibody complexes. III. Various antigen-antibody systems and the probable role of complement. J. Immunol., LXXXIII, 105-115, 1959.

58. Jensen, E. and Stetson, C. A., Humoral aspects of the immune response to homografts. II. Relationship between the hemagglutinating and cytotoxic activities of certain isoimmune sera. J. Exp. Med., CXIII, Art. 4, 785-794, 1961.

59. Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, 2nd ed., 1961, C. C. Thomas, pp. 6-7, 133-235.

60. Kalfayan, B. and Kidd, J. G., Structural changes produced in Brown-Pearce carcinoma cells by means of a specific antibody and complement. J. Exp. Med., XCVII, 145-161, 1953.

61. Kaliss, N., Immunological enhancement of tumor homografts in mice, A review. Cancer Research, XVIII, 992-1002, 1958.

62. Latta, H. and Kutsakis, A., Cytotoxic effects of specific antiserum and 17-hydrocorticosterone on cells in tissue culture. Lab. Invest., VI, 12-27, 1957.

63. Lawrence, H. S., Homograft sensitivity. An expression of the immunologic origins and consequence of individuality. Physiol. Rev., XXXIX, 811-859, 1959.

64. Levy, A. L., Complement levels in mammals. Proc. Soc. Exp. Med. Biol., XCIX, 584-585, 1958.

65. Lieberman, R., Douglas, J. O. A. and Montel, N., Production in mice of ascites fluid containing antibodies by staphylococcus or salmonella-adjuvant mixtures. J. Immunol., LXXXIV, 514-529, 1960.

66. Loud, A. V., Barany, W. C. and Albert, S., Ultrastructure of cytodes in spleen. Electron Microscope Society of America, Twenty-first Annual Meeting, August, 1963, Abst. B32.

67. Lumsden, T., On cytotoxins lethal to nucleated mammalian cells normal and malignant. Amer. J. Cancer, XXXI, 430-440, 1937.

68. Mayer, M. M., On the destruction of erythrocytes and other cells by antibody and complement. Cancer Research, XXI, 1262-1269, 1961.

69. Mayer, M. M., Osler, A. G., Bier, O. G. and Heidelberger, M., Quantitative studies on complement fixation. I. A method. J. Immunol., LIX, 195-206, 1948.

70. McCulloch, E. A. and Till, J. E., The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Rad. Res., XIII, 115-125, 1960.

71. McCulloch, E. A. and Till, J. E., The sensitivity of cells from normal mouse bone marrow to gamma radiation in vitro and in vivo. Rad. Res., XVI, 822-832, 1962.

72. McCulloch, E. A. and Till, J. E., Repression of colony forming ability of C57Bl hematopoietic cells transplanted into non-isologous hosts. J. Cell. Comp. Physiol., LXI, No. 3, 301-308, 1963.

73. McGhee, R. B., Presence of complement in serum of the mouse. Proc. Soc. Exp. Med. Biol., LXXX, 417-420, 1952.

74. McGregor, D. D. and Gowans, J. L., The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct. J. Exp. Med., CXVII, No. 2, 303-320, 1963.

75. Medawar, P. B., Reactions to homologous tissue antigens in relation to hypersensitivity. Cellular and Humoral Aspects of the Hypersensitive State, ed. H. S. Lawrence, 1959, pp. 504-529.

76. Merrill, J. P. and Hanau, C., A new technique for the study of cytotoxic antibody in vivo. Ann. N. Y. Acad. Sci., XCIX, Art. 3, 596-601, 1962.

77. Merrill, J. P., Hanau, C. and Hawes, M. D., A demonstration of a cytotoxic effect in vitro following the rejection of skin grafts by the rabbit. Ann. N. Y. Acad. Sci., LXXXVII, Art. 1, 266-272, 1960.

78. Miller, D. G., and Hsu, T. C., The action of cytotoxic antiserum on the HeLa cells of human carcinoma. Cancer Research, XVI, 306-312, 1956.

79. Miller, J. et al., An assay of graft-host interactions across strong and weak histocompatibility barriers in mice, J. Exp. Med., CXVII, Art. 5, 863-878, 1963.

80. Möller, E. and Möller, G., Quantitative studies of the sensitivity of normal and neoplastic mouse cells to the cytotoxic action of isoantibodies. J. Exp. Med., CXV, No. 3, 527-553, 1962.

81. Möller, G., Studies on the development of isoantigens of the H-2 system in newborn mice. J. Immunol., LXXXVI, No. 1, 56-68, 1961.

82. Möller, G., Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. J. Exp. Med., CXIV, 415-432, 1961.

83. Möller, G., Phenotypic expression of isoantigens of the H-2 system in embryonic and newborn mice. J. Immunol., XC, No. 2, 271-279, 1963.

84. Möller, G. and Möller, E., Studies in vitro and in vivo of the cytotoxic and enhancing effect of humoral isoantibodies, Ann. N. Y. Acad. Sci., XCIX, No. 3, 504-530, 1962.

85. Moore, R. D., Lockman, M. E. and Schoenberg, M. D., Cellular aspects of the action of Freund's adjuvant in the spleen and lymph nodes. Br. J. Exp. Path., XLIV, No. 3, 300-311, 1963.

86. Nevin, J. E. et al., A homotransplantation cytotoxin: its specific in vitro immunological activity. Surg. Forum, XIV, 152-154, 1963.

87. Oda, M. and Puck, T. T., The interaction of mammalian cells with antibodies. J. Exp. Med., CXIII, 599-610, 1961.

88. Osler, A. G., Quantitative studies of complement fixation. Bacteriol. Rev., XXII, 246-266, 1958.

89. Osler, A. G. and Heidelberger, M., Quantitative studies of complement fixation. IV. Homologous and cross-reactions in chicken and duck egg-albumin systems. J. Immunol., LX, 327-337, 1948.

90. Owen, R. D., Methods in mammalian immunogenetics. Methodology in Mammalian Genetics, 1963, Holden-Day Inc., San Francisco, pp. 347-379.

91. Palm, J., Immunogenetic aspects of tissue transplantation. Transplantation of Tissues and Cells, ed. Billingham, R. E. and Silvers, W. K., Wistar Institute Press, Philadelphia, 1961, pp. 113-132.

92. Pikovski, M., Witz, I. and Tenenbaum, E., Cytotoxic action of anti-urine sera on mouse tumor cells. Nature, CXCVIII, No. 4887, 1314-1316, 1963.

93. Pizarro, O. et al., The distribution in the tissues and the development of H-2 antigens in the mouse. Proc. Nat. Acad. Sci., XLVII, 1900-1907, 1961.

94. Pizarro, O., Rubinstein, P. and Hoecker, G., Properties of histocompatibility - 2 antigens from different tissues. Guy's Hospital Reports, CXII, No. 3, 392-401, 1963.

95. Raffel, S., Immunity, 2nd ed., 1961, Appleton-Century-Crofts, Ltd., pp. 55-170.

96. Reif, A. E., Immune cytolysis of three mouse ascites tumors. J. Immunol., LXXXIX, No. 6, 849-860, 1962.

97. Reif, A. E. and Norris, H. J., A system for quantitative determination of cytotoxic activity of antisera to ascites tumor cells. Cancer Research, XX, 1235-1244, 1960.

98. Rice, C. E. and Crowson, R. N., The interchangeability of the complement components of different animal species. II. In the hemolysis of sheep erythrocytes sensitized with rabbit amboceptor. J. Immunol., LXV, 201-210, 1950.

99. Rosenau, W. and Moon, H. D., Lysis of homologous cells by sensitized lymphocytes in tissue culture. J. Nat. Cancer Inst., XXVII, 471-477, 1961.

100. Rosenau, W. and Moon, H. D., Effect of splenic homografts on homologous cells in vitro, Lab. Invest., XI, 1260-1265, 1962.

101. Sacks, L. and Feldman, M., Cytotoxic antibody in the homograft reaction. J. Nat. Cancer Inst., XXI, 563-578, 1958.

102. Scathorne, R. J., Studies on the response of the regional lymph node to skin homografts. Ann. N. Y. Acad. Sci., LXIV, No. 5, 1028-1039, 1957.

103. Schrek, R., A method for counting the viable cells in normal and malignant cell suspensions. Amer. J. Cancer, XXVIII, 389-392, 1936.

104. Snell, G. D., Histocompatibility genes of the mouse. I. Demonstration of weak histocompatibility differences by immunization and controlled tumor dosage. J. Nat. Cancer Inst., XX, No. 4, 787-824, 1958.

105. Stavitsky, A. B., Micromethods for the study of proteins and antibodies. J. Immunol., LXXII, 360-367, 1954.

106. Stern, K. and Davidsohn, I., Heterohemo-antibodies in inbred strains of mice. I. Natural agglutinins for sheep and chicken red blood cells. J. Immunol., LXXII, 209-215, 1954.

107. Stetson, C. A., The role of humoral antibody in the homograft reaction. Advances in Immunology, III, 1963, Academic Press, New York and London, pp. 97-130.

108. Stetson, C. A. and Jensen, E., Humoral aspects of the immune response to homografts. Ann. N. Y. Acad. Sci., LXXXVII, Art. 1, 249-257, 1960.

109. Taylor, H. E. and Culling, C. F. A., Cytopathic effects in vitro of sensitized homologous and heterologous spleen cells on fibroblasts. Lab. Invest., XII, No. 9, 884-894, 1963.

110. Terasaki, P. I. et al., Antibody response to homografts. V. Cytotoxic effects upon lymphocytes as measured by time lapse cinematography. Ann. N. Y. Acad. Sci., LXXXVII, Art. 1, 258-265, 1960.

111. Terasaki, P. I. et al., Antibody response to homografts. VI, In vitro cytotoxins produced by skin homografts in rabbits. Proc. Soc. Exp. Biol. Med., CVI, 133-136, 1961.

112. Terasaki, P. I. et al., Antibody response to homografts. VII, The role of complement in cytotoxicity of serum from homografted rabbits. J. Immunol., LXXXVII, 39-45, 1961.

113. Terasaki, P. I. et al., Antibody response to homografts. VIII. Relation of mouse hemagglutinins and cytotoxins. J. Exp. Med., CXVII, No. 4, 675-690, 1963.

114. Terasaki, P. I. and Rich, N. E., Quantitative determinations of antibody and complement directed against lymphocytes. J. Immunol., XCII, No. 1, 128-138, 1964.

115. Till, J. E. and McCulloch, E. A., A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Rad. Res., XIV, 213-222, 1961.

116. Trentin, J. J. and Fahlberg, W. J., An experimental model for studies of immunologic competence in irradiated mice repopulated with "clones" of spleen cells. Conceptual Advances in Immunology and Oncology, 1962, Hoeber Medical Division, Harper and Row, Inc., pp. 66-72.

117. Uphoff, D. E. and Law, L., Genetic factors influencing irradiation protection by bone marrow. II. The H-2 locus. J. Nat. Cancer Inst., XX, No. 3, 617-632, 1958.

118. Voisin, G. A. and Maurer, P., Studies on the role of antibodies in the failure of homografts. Ann. N. Y. Acad. Sci., LXIV, Art. 5, 1053-1070, 1957.

119. Weigle, W. O. and Maurer, P., The effect of complement on soluble antigen-antibody complexes. J. Immunol., LXXIX, 211-222, 1957.

120. Wilson, R. E. and Crosby, D. L., Homotransplantation antibodies in lymphoid tissues. I. Cytotoxic activity of subcellular fractions from sensitized mice. Ann. N. Y. Acad. Sci., XCIX, Art. 3, 588-595, 1962.

121. Wilson, G. S. and Miles, A. A., Topley and Wilson's Principles of Bacteriology and Immunity, I, 4th ed., Ed. Edward Arnold Ltd., 1955, pp. 221-291.

122. Winn, H. J., Immune mechanisms in homotransplantation. I. The role of serum antibodies and complement in the neutralization of lymphoma cells. J. Immunol., LXXXIV, 530-538, 1960.

123. Winn, H. J., Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. J. Immunol., LXXXVI, 228-239, 1961.

124. Winn, H. J., The participation of complement in isoimmune reactions. Ann. N. Y. Acad. Sci., CI, Art. 1, 23-45, 1962.

125. Woodruff, M. F. A., Cellular and humoral factors in the immunity to skin homografts: Experiments with a porous membrane. Ann. N. Y. Acad. Sci., LXIV, Art. 5, 1014-1026, 1957.

126. Zaalberg, O. B. and Van Bekkum, D. W., Continued proliferation of transplanted rat lymphoid cells in irradiated mice. Transpl. Bull., VI, No. 1, 91-93, 1959.